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THE

BIOLOGICAL BULLETIN

PUBLISHED BY
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delete William F. Harrington

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II. Instructors

delete Frank E. Round

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add Stephen C. Brown, University of Michigan

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delete Barry M. Heatfield
add Donald C. Gordon, University of Rhode Island
add Conrad Gebelein, The Johns Hopkins University

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Hall, Barbara Sue, College of St. Mary of the Springs
Hine, Charles Risk, Lafayette College
Hunter, William Bruce, University of California, Santa Barbara
Langreth, Susan Grant, University of Chicago
Nutt, John Gordon, Jr., Rice University
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Walters, David Royal, Harvard University
Whisnant, Betty Lynn, Duke University

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THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised August 15, 1963)

I. The members of the Corporation shall consist of persons elected by the Board of trustees.

II. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees *ex officio,* who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(C) Trustees *Emeriti,* who shall be elected from present or former Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeriti* shall have all the rights of the Trustees, except that Trustees *Emeriti* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected for a term of five years and shall serve until his successor is selected and qualified, except that such term shall not run beyond the Annual Meeting of the Board following his 65th birthday; candidates over the age of 65 shall be elected on an annual basis. They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them except those chosen by the members, at any time. They may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These bylaws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the bylaws will be acted upon.

RESOLUTIONS ADOPTED AT TRUSTEE MEETING AUGUST 16, 1963—EXECUTIVE COMMITTEE

I. RESOLVED:

(A) The Executive Committee is hereby designated to consist of ten members as follows: *ex officio* members who shall be the Chairman of the Board of Trustees, President, Director and Treasurer; six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term.

(B) The President shall act as Chairman of the Executive Committee and the Chairman of the Board of Trustees as Vice Chairman. A majority of the members of the Executive Committee shall constitute a quorum and a majority of those present at any properly held meeting shall determine its action. It shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine.

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by the Board or by Law.

(D) The Executive Committee shall keep appropriate minutes of its meetings, and its actions shall be reported to the Board of Trustees.

II. RESOLVED:

The elected members of the Executive Committee shall be constituted as a standing "Committee for the Nominations of Officers," responsible for making nominations at the annual meeting of the Corporation and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire. (Chairman of the Board, President, Director, Treasurer, and Clerk.)

IV. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory

Gentlemen:

I submit herewith the report of the seventy-seventh session of the Marine Biological Laboratory.

1. Facilities Developments

During the past year a number of changes and developments in our facilities were completed under a grant provided by the National Science Foundation. Over the winter of 1963-64 the library stacks were repainted and fluorescent lighting installed which brightened up the stacks most satisfactorily. The glass floors in the stacks were overlaid with plywood, on top of which rubber tile was installed, getting away from the hazard of breaking of the glass flooring and also giving a quiet walking surface. New quarters were provided for the library staff, directly off the card catalogue room. The reading room has been extended to include the area formerly occupied by the library staff, providing additional space for reference material as well as additional reading space.

A centralized instrument laboratory was provided by remodeling rooms 109 and 110 in the Lillie Building where large pieces of apparatus for the general use of investigators and students can be supervised by a qualified technical assistant. A suite of laboratories centrally located on the second floor of the Lillie Building has been remodeled for electron microscopy. Included is a general service laboratory, facilities for two electron microscopists, and darkrooms for loading and developing film. These laboratories and instruments are under the supervision of an expert electron microscopist.

Also, under the National Science Foundation Grant a new collecting boat, the *Ciona,* was constructed, a 40-foot vessel powered by 180-hp. General Motors diesel engine. This boat has proved to be exceptionally seaworthy and well adapted to the Laboratory's service. The vessel was built by the Brownell Boatyard of Mattapoisett, after a design by Eldredge-McInnis, naval architects.

The old *Cayadetta* dock, extending 140 feet out into Great Harbor in front of the Laboratory, was rebuilt and was used daily throughout the summer by the *Cap'n Bill III,* which unloaded its catch of bottom fish and squid directly into mobile sea water tanks on the dock. These facilities provided by the National Science Foundation Grant have contributed significantly to improved operations in these areas. In addition the Laboratory made important modification of two of the boats to better adapt them for biological collecting. The cruising speed of the *Limulus* has been stepped up from 10 to 16 knots, extending her range. Also, a new mast and boom, operating off of a motor driven winch, has improved the operation significantly. Extensive modifications in the interior arrangements of the *Limulus* and *Dolphin* have contributed to the ease, effectiveness, and safety in the operation of these two boats.

2. *Ford Foundation Grant*

In 1963 the Planning Committee was instructed by the Executive Committee to explore ways of funding its long-range plans which had previously been accepted by the Executive Committee. This plan included additional housing for investigators and students, new laboratory facilities for the courses and a laboratory-equipped survey boat for the Systematics-Ecology Program. The needs of the Laboratory were spelled out in detail and a request for a grant of $2,500,000 toward this program was made to The Ford Foundation. The plan envisions an instructional building which will provide quarters for the regular courses and the Systematics-Ecology Program, a building of 65,000 square feet to be built at an estimated cost of $2,700,000. Also included in the program is a dining hall-dormitory to replace the present dining hall and the old residences currently used to house students. Included in the dormitory will be 125 double bedrooms. The estimated cost of this facility is $1,700,000. Also in the grant request was $200,000 for additional cottages in the Devil's Lane tract and $100,000 for the Systematics-Ecology biological survey boat.

We were delighted to receive notification in June of a grant of $2,500,000 by The Ford Foundation to the Laboratory. Of this, $300,000 is an outright grant in the amounts requested for additional cottages, particularly for younger investigators, and also funds for the Systematics-Ecology survey vessel. The remainder of the grant, $2,200,000, covers half the estimated cost of the instructional building and the dining hall-dormitory, so matching funds must be obtained in a like amount.

A topographical survey of the free area of the Devil's Lane Tract was immediately made, access roads laid out and construction of cottages promptly started. Some additional funds were obtained from individual subscribers, so that 24 cottages have been completed and will be ready for summer occupancy in 1965. The contributors included Mrs. E. Newton Harvey, Mrs. Gary N. Calkins, Mrs. Samuel O. Mast and The Grass Foundation. Also, an extensive play area, readily accessible to both cottage colonies, has been laid out, providing recreation facilities for children of all ages as well as adults.

Plans for the new Systematics-Ecology survey vessel have been completed and construction will soon be started. This vessel will be 65 feet long, of steel construction and equipped with the most modern equipment and gear for survey work.

Plans are being developed by the firm of Pierce, Pierce and Luykx for the instruction building and the dining hall-dormitory. The instruction building will be located on the north side of Center Street, east of the Apartment House. The site for the dining hall-dormitory has not yet been selected. In the meantime the Planning Committee is exploring various sources of additional funds to match the grant from The Ford Foundation toward these two facilities.

3. *Richard K. Mellon Foundation Grant*

The Laboratory was most fortunate in receiving a grant of $50,000 from the Richard K. Mellon Foundation, toward the cost of construction of the instruction building. We were very much gratified by this support from one of our Woods Hole neighbors.

4. *Rand Bequest*

The Laboratory was the beneficiary of a bequest this past year by the will of Mrs. Herbert W. Rand, in memory of her husband, Professor Herbert W. Rand, of Harvard University. Professor Rand first came to the Marine Biological Laboratory in 1923, and became a member of the Corporation in 1928. He resided in Falmouth after his retirement at Harvard in 1942 until his death.

5. *Personnel*

Deborah Lawrence Harlow was a member of the Library staff at the Marine Biological Laboratory for 40 years, retiring at the end of 1964. She joined the staff in 1925 as secretary to the Librarian. Mrs. Harlow became thoroughly acquainted with the operation of the Library over a period of years and succeeded Mrs. Montgomery as Librarian in 1948. During her tenure as Librarian, the number of journals to which the Laboratory subscribed increased from 1200 to 1717, the number of volumes in the Library from 56,000 to well over 100,000. Although these are striking increases in numbers, Mrs. Harlow will always be remembered for her very effective management of the Library, for her cooperativeness and for the relaxed manner in which she furthered the library work of the scientists and students at the Laboratory. Mr. Harlow, for 17 years head of the machine shop, and expert in his field, retired at the same time.

1. Memorials

WINTHROP JOHN VANLEUVEN OSTERHOUT

By Theodore Shedlovsky

On August 2, 1871, Winthrop John Vanleuven Osterhout was born in Brooklyn, New York, the son of a Baptist minister whose ancestors came to America in the seventeenth century from the town of Osterhout in Holland. On April 9th of this year, Dr. Osterhout died in New York in his ninety-third year. In accordance with his wishes his ashes are buried in the cemetery of the Church of St. James the Less in Philadelphia, among four descendants of Benjamin Franklin, an ancestor of his widow, Marian Irwin Osterhout. Many of us who knew him personally, and the Marine Biological Laboratory, where he spent well over half the summers of his long life, mourn his death. It seems to mark the passing of an important period in the history of biological science, a period which bridged the nineteenth and twentieth centuries. At Woods Hole, as elsewhere, quantitative experimentation and important new ideas were supplementing or displacing the traditional descriptive methods of research in biology.

Here, at the Marine Biological Laboratory, a number of dedicated biologists, who were already eminent or were soon to become so, carried on their researches during the summer and influenced students—an enterprise which is happily continuing. Among these dedicated biologists we find the names of Jacques Loeb, T. H. Morgan, Frank and Ralph Lillie, E. B. Wilson, E. G. Conklin, Walter Garrey, A. P. Mathews, Ivey Lewis, and, of course, W. J. V. Osterhout. Let us examine briefly his history as a man of science.

While still an undergraduate student at Brown University, in 1892 young Osterhout came to the Marine Biological Laboratory, where W. A. Setchell introduced him to research in botany. He started work and soon found that the four spores in a red alga, *Aquardhiella tenera*, each of which could produce a new plant, were able to combine and form a single plant. A year later, Osterhout had received the A.B. degree from Brown (I believe he was the class poet) and was again at Woods Hole, but now as Setchell's assistant in the Botany Course. Together, while collecting in Nobska Pond, they found *Nitella*, but physiological experiments on this interesting material came only considerably later.

After a year in Bonn, Germany, with the eminent plant cytologist, Eduard Strasburger (1895–6) Setchell brought Osterhout to the University of California where he earned the Ph.D. degree in 1899 and met Jacques Loeb in 1902. Learning of Loeb's work on animal cells he began to make similar studies on plant cells and did so with considerable success. Among other things, he was much interested in Loeb's observations on ionic antagonism, such as exists between monovalent and divalent or trivalent cations, and he used effectively the measurement of electrical conductance in such experiments with plant cells. Acquaintance with Loeb soon ripened into a great, life-long friendship. While still in California, Osterhout got to know Hugo de Vries and Svante Arrhenius. There, in those early years of the century, he doubtless participated in many discussions of matters scientific, philosophic, as well as honestly convivial.

In 1909, Osterhout left the University of California as Associate Professor and moved to Harvard as Assistant Professor, to become Professor in 1913. When Loeb, who was a member of The Rockefeller Institute for Medical Research, died in 1924, Dr. Osterhout was invited by the Director, Dr. Flexner, to accept membership in the Institute. This invitation he accepted. At the Institute he was given a substantial department of general physiology and a small laboratory in Bermuda for work on *Valonia* and *Halicystis*. In New York he was joined by Drs. Marian Irwin, Lawrence Blinks, and, a little later, by S. E. Hill, W. Stanley and several others. Interested, as he always was, in a physico-chemical approach to biological problems he arranged for D. A. MacInnes to form a physical-chemical group, affiliated with his department. L. G. Longsworth and I soon joined that group.

After his return east from California, and while he was still at Harvard, Osterhout again became intimately associated with the Marine Biological Laboratory and remained so until just a few years ago. He had been a Trustee since 1910. Those of us who had the privilege of knowing him here at Woods Hole, in Cambridge, or in New York, will fondly recall scientific discussions with him, which often took the form of Socratic dialogues, general conversations which were seldom trivial and were usually well seasoned with wit and wisdom. We remember him as a gentleman, in the best and most accurate sense of the word, always with dignity but never with pomp or without a subtle warmth. We shall miss him; not only the scientist, botanist, physiologist, but also the mentor, the councillor, the friend. I speak not only for myself but also, I am certain, for many others.

Osterhout was a superb teacher. Although I did not have the good fortune of being one of his students at Harvard, I know that his influence in attracting young people to research in biology was great. He had a gift for devising beautiful experimental demonstrations which were presented with a persuasive but dignified enthusiasm for the subject that inspired many of his graduate students to undertake productive careers in research.

What were his main contributions to science? Here in the Marine Biological Laboratory Library, there are about 270 cards in the W. J. V. Osterhout file. These include references to his early work in cytology, salt antagonism, osmotic studies and other physico-chemical aspects of plant cells and plant cell models. Perhaps his principal work was on permeability aspects and electrical properties of single plant cells. He was very early in accounting for the active transport of ions by a molecular carrier mechanism. To show this he constructed cell models which exhibited active transport with carrier molecules passing through non-aqueous membranes. For example, aqueous tricholoracetic acid and pure water, separated by a layer of guaiacol in the bottom of a U-tube, showed the water apparently moving against its chemical potential gradient. Water movement and water absorption interested him greatly and formed one of the subjects of his work with Mrs. Osterhout into the evening of his scientific life.

Beginning with his early experiments in California on the relation of electrical conductivity of plant cells and ionic antagonisms, the bio-electric phenomena in living cells had always held his active interest. This traditionally controversial field has been so from the time of Volta and Galvani, through the period of phase boundary potentials *versus* diffusion potentials, and even remains so today in the present era of biochemical and biophysical euphoria. Such a field is not an easy one to explore, but as Osterhout said of Loeb, "He did not select problems because they were easy, but because of their importance. His courage sprung largely from his faith in the materialistic conception. . . ."

While at Harvard, his extensive electrical studies on *Laminaria* led in 1922 to the book, *"Injury, Recovery and Death in Relation to Conductivity and Permeability."* This book stimulated other investigators by its novelty of concept and method of interpretation involving consecutive reactions. Throughout his life, Osterhout stimulated biologists to engage in meaningful quantitative experiments, and physical chemists and physicists to consider the problems presented by the living cell.

Questions of photosynthesis, respiration, oxidation and related topics had received attention in his publications. In particular, mention may be made of his demonstration of photo induction through a striking observation with A. R. Haas (1918) in which he noted that when the marine plant, *Ulva,* was transferred from darkness to light the rate of photosynthesis was increased.

I have already mentioned the important concept of carrier molecules, so much invoked today. It should also be noted that Osterhout pioneered in the concept of the steady-state as against equilibrium in accounting for the kinetics of penetration of substances into living cells, and, of course, no self-respecting student of molecular biology today will deny at least some knowledge of irreversible thermodynamics. But Osterhout's influence in general physiology was even greater than the sum of his papers and of his personal contacts with other investigators and students. I refer to the *Journal of General Physiology.* He was, with Jacques Loeb, co-editor of this journal from its beginning. Let me quote from his own words in the *"Outline of the History of the Journal of General Physiology,"* written in 1955: "Dr. Jacques Loeb and I realized the need for a journal to promote the study of general physiology. Dr. Flexner agreed to publish the Journal from The Rockefeller Institute for Medical Research beginning in 1918. Dr. Loeb and I were the sole editors until he died in 1924. The statement, 'Founded by Jacques Loeb' was placed on the cover of the Journal and a memorial volume was published in his honor. Dr. John H. Northrop and Dr. William J. Crozier joined the editorial board in 1924 after Loeb's death. For about twenty-two years Dr. Northrop, Dr. Crozier and I were the only editors. In 1946 Dr. Wallace O. Fenn joined us. For about thirty-seven years each editor read every paper submitted."

We shall miss Dr. Osterhout. But, for many of us, memory will often be refreshed when we see the *Journal of General Physiology,* when we visit the Marine Biological Laboratory, when we think of *Nitella, Valonia, Halicystis, Laminaria,* or when we recall the wit and wisdom which so often emanated from him to inspire us in so many ways.

ELIOT R. CLARK

By Sears Crowell

Dr. Eliot R. Clark was born November 13, 1881, in Shelburne, Massachusetts. He received his A.B. degree from Yale University in 1903, and the M.D. degree from The Johns Hopkins University Medical School in 1907. He was on the staff of the Department of Anatomy at The Johns Hopkins University and carried on postdoctoral studies at the Universities of Munich and Krakow. From 1914 to 1922 he was a professor at the University of Missouri, and from 1922 to 1926 at the University of Georgia. In 1926 he became Head of the Department of Anatomy at the University of Pennsylvania, a post which he held until 1947. He became Professor *Emeritus* in 1950 and a guest investigator at the Wistar Institute. He received an honorary Sc.D. from Washington and Jefferson College in 1940. During the past seven years a serious heart condition prevented much physical activity but he retained an alert mind and lively interest in the affairs of Woods Hole. His death came instantly on November 1, 1963 in his home in Philadelphia.

His association with the Marine Biological Laboratory began in 1909 when he became a member of the Corporation and also met Eleanor Linton, who was to become his wife two years later. He served as a Trustee of the Laboratory from 1930 to 1946.

Mrs. Clark's father was Dr. Edwin Linton, a distinguished parasitologist, who worked at the Bureau of Fisheries. Dr. and Mrs. Linton devoted much time and attention to the Marine Biological Laboratory Club and the Clarks carried on this tradition. Dr. Clark served as Secretary-Treasurer in 1918 and 1919, and later as President. Both of the Clarks were active in various fund-raising affairs for the Marine Biological Laboratory Club and Tennis Club.

In the Woods Hole community the Clarks were involved with the choral group and in support of the Penzance Players. Later, as their children became sailing enthusiasts, the Clarks contributed much to the Woods Hole Yacht Club. Dr. Clark was acting Commodore during World War II, and Commodore from 1947 to 1950.

Apart from his connections with Woods Hole, Dr. Clark was most active professionally in the American Association of Anatomists, serving as their Secretary-Treasurer for the periods of 1938–1942 and 1943–1946. He was largely instrumental in building up the excellent collection of research motion pictures, now housed in the Wistar Institute. He served for many years as Chairman of the Committee on Motion Pictures of the American Association of Anatomists. He reviewed films submitted by investigators and wrote brief descriptions of each accepted. He compiled the lists of these for publication in the *Anatomical Record.*

The majority of Eliot Clark's papers, many of them with Mrs. Clark as co-author, deal with problems of the circulatory system. Although he regarded himself as an anatomist, much of his work is developmental and physiological. By about 1930 he and his associates had perfected the technique of implanting windows in the ears of rabbits. With this technique he was able to study microscopically the development of blood vessels, lymphatics, nerves, epidermis, and various implanted tissues. Older workers at the Marine Biological Laboratory will recall with pleasure the demonstrations presented by the Clarks at the scientific meetings of the Laboratory.

Dr. Clark's careful attention to detail, thoroughness, and devotion characterized both his scientific work and his services to the scientific community, the Marine Biological Laboratory, and Woods Hole.

FRANK PATTENGILL KNOWLTON

By Walter S. Root

Frank P(attengill) Knowlton, the son of Charles Fox and Mary (Pattengill) Knowlton, was born in Hollard Patent, New York, on June 17, 1875. He received the A.B. degree from Hamilton College in 1896 and the M.A. degree from the University of Michigan in 1897. From 1897–1900 he was an Instructor in Physiology and Embryology at the College of Medicine, Syracuse University. During this period he also studied medicine, receiving the M.D. degree in 1900. He served successively at Syracuse as Lecturer in Physiology, 1900–1906; Associate Professor, 1906–1908; Professor 1908–1946; and *Emeritus* Professor of Physiology since 1946. As student and teacher he spent 49 years at Syracuse. I remember his remarking one day as he surveyed a new class that teaching the sons of former students can be taken in one's stride, but that when the grandsons appear, one feels older.

Dr. Knowlton spent the years of 1911–1912 at Cambridge University and at University College, London. The studies carried out at this time were concerned with the relation of colloids to diuresis, the effects of stimulating the 8th and 9th spinal nerve roots upon the toad bladder, the sugar consumption of the isolated mammalian heart, the sugar consumption of the normal and diabetic heart, and the nature of pancreatic diabetes. They were published in four papers in the *Journal of Physiology*, one in the *Proceedings of the Royal Society* and one in the *Zeitschrift für Physiologie.* Of this work, perhaps the most important was the development and use of the heart-lung preparation in collaboration with the great physiologist, Ernest H. Starling. Knowlton was fortunate in having the opportunity of working in England at a time when an impressive group of men were active. Among these may be mentioned F. Gowland Hopkins, John Scott Haldane, Charles Sherrington, Joseph Barcroft, John Newport Langley, T. R.

Elliott, William Bayliss, Walter Fletcher, E. A. Sharpey-Schafer, James Mackenzie, Henry Head, Thomas Lewis and others.

Knowlton maintained a continuing interest in renal physiology, carbohydrate metabolism and *diabetes mellitus.* In the latter part of his professional life he was active in studying the physiology of inhibition. The published papers on this subject are concerned with reciprocal inhibition in the earthworm, peripheral inhibition in arthropods, peripheral neuromuscular augmentation in the heart of *Limulus polyphemus,* inhibition in the cardiac ganglia of *Limulus,* the dual control of crustacean muscle, and inhibition of the turtle heart, and of turtle atria.

Many of the publications in the field of comparative physiology were carried out in the Marine Biological Laboratory of which he was a Trustee from 1932 to 1946, and a Trustee *Emeritus* since 1946. As in his English experience, Knowlton was active in Woods Hole at a time when the Laboratory housed many distinguished scientists.

In 1902 Dr. Knowlton married Clara Avis Roberts. It was a congenial partnership, and their home was always a pleasant place to visit. They had one child, a daughter, Catherine Morilla (now Mrs. Lucius Foote), who like her father is a medical graduate of Syracuse. A grandson, Knowlton Foote, is currently a graduate student in Biochemistry at the Syracuse Medical Center.

Dr. Knowlton was a member of the Physiological Society of Great Britain, and the American Physiological Society, the annual meeting of which he attended regularly. He was active in the Western New York Section of the Society for Experimental Biology and Medicine for many years. He was also a member of a number of social and honorary societies (Delta Upsilon, Nu Sigma Nu, Sigma Xi, Alpha Omega Alpha, Phi Kappa Phi).

Dr. Knowlton was a modest man. He was always a kind and considerate companion. A mutual friend wrote to me recently that he had died on October 30, 1963, without pain or suffering. I liked the statement that his end was much as his life had been, peaceful and orderly.

IVEY FOREMAN LEWIS

By Carl C. Speidel

Ivey Foreman Lewis was born on August 31, 1882, in Raleigh, North Carolina. Eighty-one years later he died in Charlottesville, Virginia, on March 16, 1964, after having been associated with the University of Virginia for nearly 50 years. He is survived by a son, Ivey Foreman Lewis, Jr., of Hampton, and a daughter, Margaret Elliott Lewis of Charlottesville.

Ivey Lewis graduated from the University of North Carolina, receiving the degrees of A.B. in 1902 and M.S. in 1903. He was awarded the Ph.D. degree by The Johns Hopkins University in 1908. For his published dissertation, entitled *"The Life History of Griffithsia bornetiana,"* he was given the Walker Prize by the Boston Society of Natural History. He studied abroad in 1908 at the University of Bonn and at the Naples Zoological Station. During the academic year 1905–1906 and again after returning from Naples, he was Professor of Biology at Randolph-Macon College at Ashland, Virginia. During this period he made the acquaintance of Margaret Hunter, of Ashland, who became his wife in 1909. Three years later he left Randolph-Macon College to be Assistant Professor of Botany at the University of Wisconsin. In 1914 he was made Professor of Botany at the University of Missouri. The following year he was appointed Miller Professor of Biology and Agriculture at the University of Virginia, where he served as Professor, and also as Dean from 1934 on, until his retirement in 1953.

For many years Dr. Lewis was associated with the Woods Hole Marine Biological Laboratory. He was an Instructor in Botany in 1907 and again from 1910 through

1917. From 1918 through 1927 he was in charge of botanical instruction, and also served as Trustee and member of the Executive Committee. In 1928 he was a Carnegie Fellow at the Dry Tortugas Laboratory and in 1929 a Professor at the Hopkins Marine Station of Stanford University. From 1933 until 1946 he was Director of the University of Virginia's newly established summer station at Mountain Lake, Virginia.

Dr. Lewis founded the Association of Virginia Biologists in 1920. This gave rise to the Virginia Academy of Science in 1923. The following year he was elected its first President. For eight years he was a member of the Division of Biology and Agriculture of the National Research Council and served as Chairman from 1933 through 1936. He was President of the American Society of Naturalists (1939), President of the American Biological Society (1942), and President of the Botanical Society of America (1949).

In 1947 he received an honorary degree of Doctor of Science from the University of North Carolina. In 1959, six years after his retirement from active duty, he was the recipient of the University of Virginia's Thomas Jefferson Award.

Dr. Lewis's research interests were primarily in the field of algology. In addition to his prize-winning doctorate thesis cited above, his publications include papers dealing with the algae of the Woods Hole and Charlottesville regions, the vegetation of Shackleford Bank, North Carolina, and the pollen of the Dismal Swamp of Virginia and North Carolina.

One of his papers, entitled "The Flora of Penikese, Fifty Years After," published in *Rhodora* in 1924, is of historic interest to the Marine Biological Laboratory. This is a survey similar to one made in 1873 by David Starr Jordan, a member of Agassiz's Laboratory which was located on Penikese Island. Jordan's results were published in the *American Naturalist* in 1874. Dr. Lewis served as editor of the 1924 survey which was made cooperatively by the students and staff of the course in Botany at the Marine Biological Laboratory. Agassiz's Laboratory on Penikese is regarded as a kind of forerunner of the Woods Hole Marine Biological Laboratory.

For recreation Dr. Lewis greatly enjoyed the numerous collecting trips made as a part of the course in Botany. He also enjoyed the game of tennis and was an enthusiastic and proficient player. His figure was a familiar one on the Mess Hall court. He always regretted that his duties in connection with the Mountain Lake Station in Virginia made it necessary for him to terminate his regular summer attendance at Woods Hole.

Ivey Lewis's influence on the Marine Biological Laboratory was a beneficent one. In the most literal sense of the expression, he was a gentleman and a scholar. Those of us who knew him well miss him greatly.

2. The Staff

EMBRYOLOGY

I. Instructors

James D. Ebert, Director, Department of Embryology, Carnegie Institution of Washington, in charge of course

Donald D. Brown, Staff Member, Department of Embryology, Carnegie Institution of Washington

Allison L. Burnett, Associate Professor of Biology, Western Reserve University

Robert L. De Haan, Staff Member, Department of Embryology, Carnegie Institution of Washington

Thomas J. King, Head, Department of Embryology, Institute for Cancer Research, Philadelphia

James W. Lash, Assistant Professor of Anatomy, University of Pennsylvania
Aaron A. Moscona, Professor of Zoology, University of Chicago

II. Laboratory Assistants

C. B. Kimmel, The Johns Hopkins University
David E. Kohne, Purdue University

III. Lectures

J. D. Ebert	Perspectives in developmental biology
T. J. King	Teleosts I
T. J. King	Teleosts II
R. L. De Haan	Cell movements and morphogenesis
A. J. Coulombre	The morphogenetic interaction of the tissues of the eye during development
E. Zwilling	Morphotypic diversity *vs.* histiotypic identity
S. Simpson	Tissue interactions and morphogenesis in lizard tail regenerations
J. Lash	The induction of chondrogenesis *in vitro*
A. Moscona	Sponges I
A. Moscona	Sponges II
A. Moscona	Tissue reconstruction from dissociated cells
H. Rubin	The malignant transformation of animal cells by viruses
M. Rosenberg	Some applications of surface physics to cell biology
P. Marcus	Dynamics of plasma membrane modification in virus-infected and normal cells
A. L. Colwin	Role of the gamete membranes in fertilization
M. Steinberg	Adhesive selectivity in intercellular reactions
A. Burnett	A model of growth for hydroids and tubules of higher organisms
A. Burnett	The role of neoblasts in the maintenance of form of hydroids
A. Burnett	Dedifferentiation and redifferentiation of somatic cells in *Hydra*—an analysis of polymorphism
J. D. Ebert	Developmental aspects of immunity
J. W. Lash	Ascidians I: General embryology
J. W. Lash	Ascidians II: Metamorphosis
K. R. Porter	Developmental cytology I
K. R. Porter	Developmental cytology II
E. Hadorn	Developmental aspects of pleiotropic effects of genes
J. Gall	The nucleic acids of giant chromosomes
T. J. King	The developmental capacity of nuclei transplanted from advanced embryos
R. L. De Haan	Comparative morphogenesis of annelids, molluscs and echinoderms
E. Hadorn	Problems of differentiation and pattern formation in *Drosophila* blastemas
D. D. Brown	Biochemistry of oogenesis and early development
P. Gross E. Bell J. R. Collier I. David A. Monroy	Microsymposium: biochemistry of early development

A. B. Pardee	Cell regulatory mechanisms I
A. B. Pardee	Cell regulatory mechanisms II
S. Cohen	An epidermal growth-stimulating protein
M. Singer	The nervous control of the regeneration of body parts in vertebrates
V. Hamburger	Neurogenesis and the embryology of behavior
K. Meyer	Keratosulfates of cornea and cartilage
G. W. Cooper	Induction of somite chondrogenesis by cartilage and notochord: a correlation between inductive activity and cytodifferentiation
H. Schneiderman	The hormonal control of insect development
L. Weiss	The structure of lymphatic tissue and its reaction in runt disease

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania
Arthur K. Parpart, Professor of Biology, Princeton University
Albert Szent-Györgyi, Director, Institute for Muscle Research, Marine Biological Laboratory
W. D. McElroy, Director, McCollum-Pratt Institute, The Johns Hopkins University

II. Instructors

J. Woodland Hastings, Professor of Biochemistry, University of Illinois, in charge of course
E. A. Adelberg, Professor of Microbiology, Yale University
Harlyn Halvorson, Professor of Bacteriology, University of Wisconsin
Shinya Inoué, Professor of Cytology, Dartmouth College
K. E. Van Holde, Professor of Physical Chemistry, University of Illinois
Fred Karush, Professor of Microbiology, University of Pennsylvania
William F. Harrington, Professor of Biology, The Johns Hopkins University
Hans Kornberg, Professor of Biochemistry, Leicester University, England

III. Laboratory Assistants

George Kissil, University of Wisconsin
Carolyn Eberhard, University of California at Berkeley

IV. Lectures

Seymour Cohen	The lethality of streptomycin and the stimulation of RNA synthesis
A. J. Sophianopoulos	Protein denaturation and hydrogen-ion equilibria of lysozyme
Howard Schachman	Macromolecular configurations
George Wald	Human color vision
R. K. Clayton	Photosynthesis II: Physical aspects
Roger Eckert	Excitation-response coupling: Bioelectric flash triggering in *Noctiluca*
William Hagins	Early steps in the excitation of photoreceptors

Alex Keynan	The bacterial endospore and the problem of biological dormancy
H. E. Huxley	Fine structure of muscle
Ian Gibbons	Studies on ciliary movement
C. Veegar	Studies on the spectral complexes between flavo-proteins and their competitive inhibitors
Rachmiel Levine	Mechanism of hormone action
Albert Szent-Györgyi	Growth
Hans Leo Kornberg	Microbial metabolism I
Hans Leo Kornberg	Microbial metabolism II
Hans Leo Kornberg	Microbial metabolism III
Fred Karush	Immunochemistry I. The interactions of immunoglobulins
Fred Karush	Immunochemistry II. The nature of immunoglobulins
Fred Karush	Immunochemistry III. The biosynthesis of immunoglobulins
Lawrence Levine	Applications of immunochemistry to problems in biology
Irvin Isenberg	Phosphorescence properties of DNA complexes
Cyrus Levinthal	Synthesis and stability of messenger RNA
R. K. Clayton	Photosynthesis I: Biochemical aspects
E. A. Adelberg	Transcription and translation of the bacterial chromosome
E. A. Adelberg	Replication and transfer of bacterial DNA
K. C. Atwood	Some aspects of regulation of gene function
S. Inoué	Polarization microscopy: An approach to fine-structure analysis in living cells
S. Inoué	Dynamic aspects of the mitotic apparatus
S. Inoué	Molecular arrangement of DNA in the living sperm
Hans Leo Kornberg	Microbial metabolism
K. E. Van Holde	Protein structure III. Multichain proteins
Harlyn Halvorson	Protein biosynthesis I: *In vivo*
Harlyn Halvorson	Protein biosynthesis II: *In vitro*
Harlyn Halvorson	The control and timing of enzyme synthesis
E. A. Adelberg	The bacterial chromosome: Structure and function
E. A. Adelberg	The bacterial chromosome: Replication
J. W. Hastings	Enzymatic intermediates in the bacterial bioluminescent reaction
J. W. Hastings	Crystalline bioluminescent particles: The *Gonyaulax* system
William P. Jencks	Some aspects of the mechanism of enzyme action
K. E. Van Holde	Protein Structure I: Physical methods of investigation
K. E. Van Holde	Protein Structure II: The folding of polypeptide chains

MARINE BOTANY

I. Consultants

William Randolph Taylor, Professor of Botany, University of Michigan
Richard C. Starr, Professor of Botany, Indiana University

II. Instructors

Walter R. Herndon, Professor of Botany, University of Tennessee, in charge of course
Philip W. Cook, Department of Botany, University of Vermont
H. Wayne Nichols, Assistant Professor of Botany, Washington University
Frank E. Round, Department of Botany, University of Bristol, England
Robert T. Wilce, Assistant Professor of Botany, University of Massachusetts

III. Laboratory Assistants

Russell G. Rhodes, Department of Botany, University of Tennessee
Ernest Neal, Department of Botany, University of Tennessee

IV. Collector

Martha Hodge, University of Michigan

INVERTEBRATE ZOOLOGY

I. Consultants

F. A. Brown, Jr., Morrison Professor of Biology, Northwestern University
Libbie H. Hyman, American Museum of Natural History
Clark P. Read, Professor of Biology, Rice University
Alfred C. Redfield, Woods Hole Oceanographic Institution

II. Instructors

W. D. Russell Hunter, Professor of Zoology, Syracuse University, in charge of course
George Holz, Professor of Microbiology, State University of New York, Upstate Medical Center
Roger Milkman, Associate Professor of Zoology, Syracuse University
Irwin W. Sherman, Assistant Professor of Biology, University of California at Riverside
Allahverdi Farmanfarmaian, Professor of General Physiology, Pahlavi University, Shiraz, Iran
Eric L. Mills, Assistant Professor of Biology, Queen's University, Kingston, Ontario, Canada
Frank M. Fisher, Assistant Professor of Biology, Rice University
Sears Crowell, Professor of Zoology, Indiana University

III. Assistants

John H. Busser, University of Rhode Island
W. Bruce Hunter, University of California at Santa Barbara

IV. Lectures

Roger D. Milkman	Protochordata II
John J. Lee	The study of living Foraminifera in the laboratory
George Holz	The nature of the Protozoa
George Holz	Mastigophora
George Holz	Rhizopodea and Actinopodea
George Holz	Foraminifera
George Holz	Ciliophora
R. A. Boolootian, A. Farmanfarmaian	Dialogue on aspects of echinoderm physiology
W. D. R. Hunter	An approach to zoöplankton
A. Farmanfarmaian	The echinoderms I Introduction: Crinoidea and Asteroidea

Irwin W. Sherman	Physiological studies on malarial parasites
A. Farmanfarmaian	The echinoderms II: Holothuroidea
A. Farmanfarmaian	The echinoderms III: Echinoidea and Ophiuroidea
Roger D. Milkman	Protochordata I
Eric L. Mills	Arthropoda II: Larvae, lines and limbs—further introduction to the Crustacea
W. D. Russell Hunter	Molluscs leave the sea (physiological variation and evolution)
Eric L. Mills	Arthropoda III: Feeding in Crustacea—Cephalocarida, Branchiopoda, and Mystacocarida
Eric L. Mills	Arthropoda IV: Feeding in Crustacea—Copepoda, Cirripedia, and Malacostraca
Luigi Provasoli	External metabolites in sea water
Robert Hessler	Arthropoda V: Functional morphology of jaws and other things in the Crustacea
Eric L. Mills	Arthropoda VI: Pycnogonida and Xiphosurida
Frank Fisher	Ectoprocta and Entoprocta
Frank Fisher	Aschelminthes
W. D. Russell Hunter	Mollusca IV: Functional morphology in Cephalopoda and minor groups
Clark P. Read	Physiology of parasitic flatworms
Irwin W. Sherman	Annelida I: Introduction, reproduction and development
Irwin W. Sherman	Annelida II: Settling of larvae, regeneration, feeding and locomotion
Irwin W. Sherman	Annelida III: Respiration, osmoregulation, neuromuscular system and behavior
Eric L. Mills	Arthropoda I: Introduction to the Crustacea: The biology of limbs and exoskeleton
Roger D. Milkman	Porifera II
Frank Fisher	Platyhelminthes I: Turbellaria and Trematoda
Frank Fisher	Platyhelminthes II: Cestoda and Rhynchocoela
W. D. Russell Hunter	Mollusca I: General molluscan organization—functioning of mantle cavity in Gastropoda
W. D. Russell Hunter	Mollusca II: Gastropoda—mantle cavity and feeding mechanisms in Bivalvia
W. D. Russell Hunter	Mollusca III: Adaptations in bivalves—aspects of general physiology of Gastropods and bivalves
Sears Crowell	Cnidaria I: General diversity in hydroids
Sears Crowell	Cnidaria II: Hydroid morphogenesis
Sears Crowell	Cnidaria III: Other Cnidaria—physiology of Cnidaria
Sears Crowell	Ctenophora
Roger D. Milkman	Porifera I

MARINE ECOLOGY

I. Consultants

Melbourne R. Carriker, Marine Biological Laboratory
Bostwick H. Ketchum, Woods Hole Oceanographic Institution
Edwin T. Moul, Rutgers University
John H. Ryther, Woods Hole Oceanographic Institution

II. Instructors

W. Rowland Taylor, Department of Oceanography and the Chesapeake Bay Institute, The Johns Hopkins University, in charge of course

Howard L. Sanders, Woods Hole Oceanographic Institution

Lawrence B. Slobodkin, Department of Zoology, University of Michigan

Richard A. Boolootian, Department of Zoology, University of California at Los Angeles

Otto Kinne, Biologische Anstalt Helgoland

III. Laboratory Assistants

Margaret C. Lloyd, University of Michigan

Barry M. Heatfield, University of California at Los Angeles

IV. Lectures

W. Rowland Taylor	Organisms and their environment experimental approaches
W. Rowland Taylor	The marine environment, its chemistry and physics
W. Rowland Taylor	Phytoplankton I: Diatoms
W. Rowland Taylor	Phytoplankton II: Dinoflagellates
W. Rowland Taylor	Primary productivity by phytoplankton I
W. Rowland Taylor	Primary productivity by phytoplankton II
M. R. Carriker	The Systematics-Ecology Program at the Marine Biological Laboratory
J. H. Ryther	Geographical variations in productivity
D. Menzel	Production and utilization of dissolved organic material in the oceans
W. R. Taylor	Physiology of migrating littoral diatoms
B. H. Ketchum	Nutrient cycles in the sea
E. R. Baylor	Sea surface chemistry and the distribution of organisms
H. L. Sanders	Animal-sediment relationships
H. L. Sanders	A study of a marine benthic community
R. Scheltema	Problems in benthic larval ecology
H. L. Sanders	Salinity, hydrography and the distribution of estuarine animals
Eric L. Mills	Ecology of a crustacean sibling species pair, or systematics unashamed
J. B. Pearce	Temporal and spatial distribution of mytilid associations
S. A. Wainright	Biology of reef corals
J. A. Hellebust	Excretion of organic compounds by marine algae
Otto Kinne	The effects of temperature on marine and brackish water organisms
L. Provasoli	Culturing marine algae I: (Joint lecture with the Botany Course)
L. Provasoli	Culturing marine algae II: (Joint lecture with the Botany Course)
Otto Kinne	Effects of temperature and salinity on the hydroid, *Cordylophora caspia*
L. Provasoli	External metabolites in sea water
Otto Kinne	Effects of temperature, salinity and oxygen on the fish, *Cyprinodon macularius*

A. Farmanfarmaian	Temperature and salinity tolerance limits of the West Coast purple sea urchin
Otto Kinne	Non-genetic adaptation to temperature and salinity in marine and brackish water organisms
L. Slobodkin	Ecological tautologies
L. Slobodkin	Fecundity, mortality and reproductive value
L. Slobodkin	Experimental population growth
L. Slobodkin	Energy and animal populations
L. Slobodkin	The strategy of evolution
R. A. Boolootian	Food requirements and distribution of marine organisms: general considerations
R. A. Boolootian	Types of food utilization by marine organisms with emphasis on feeding adaptions
R. A. Boolootian	Reproductive biology of marine organisms: General patterns
R. A. Boolootian	Factors influencing the regulation of reproductive cycles
R. A. Boolootian	Reproductive physiology of the purple sea urchin, *Strongylocentrotus purpuratus*

Wednesday Evening Lecture Series, jointly sponsored by Marine Ecology, Invertebrate Zoology and Comparative Physiology:

N. D. Marshall	Some aspects of the biology of benthic deep-sea fishes
J. H. Welsh	Serotonin: Its occurrence in nature and its multiple biological roles
Carroll M. Williams	Light, brains, and metamorphosis
B. C. Abbott	Excitation-contraction coupling and mechanical responses in relation to muscle function

SYSTEMATICS–ECOLOGY PROGRAM

The Staff

Director: Melbourne R. Carriker

Resident Systematist: Victor A. Zullo

Resident Ecologist: Robert H. Parker

Postdoctoral Fellows and Research Associates: Marvin Cantor, John C. H. Carter, David C. Grant, Jack B. Pearce, Kay W. Petersen, Thomas J. M. Schopf, Joseph Simon, Edmund H. Smith, Gerald E. Walsh

Visiting Investigators in Residence: Richard A. Boolootian, Louise Bush, Duane Hope, E. T. Moul

Secretaries: San Lineaweaver, Virginia Smith

Artists: Ruth von Arx, Diane Johnson

Captain of Research Vessel: James P. W. Ostergard, Jr.

Research Assistants: Kay Cram, Andrew L. Driscoll, J. Stewart Nagle, Peter J. Oldham, Peter E. Schwamb, Dirk van Zandt, Hilary M. Williams, June Thomas, Vilia Turner

I. Seminars (winter not included)

Albert Szent-Györgyi	Contraction of muscle
John H. Ryther	U. S. Biological Program of the Indian Ocean Expedition
Victor A. Zullo	Keys to marine invertebrates of the Woods Hole region

Jack B. Pearce	A preliminary report on the *Mytilus edulis* association in Quicks Hole, Elizabeth Islands, Massachusetts
James Ross	Electrometric measurements of activities of ions and gases as applied to measurements in animals and of sea water
Joseph L. Simon	Reproduction and larval development in the spionid polychaete, *Spio setosa*
Richard A. Boolootian	Aspects of reproductive biology of echinoderms
Eric L. Mills	The biology of an amphipod crustacean sibling species pair
Walter R. Herndon	Some approaches to taxonomic revision in chlorophycean algae
Marvin Cantor	Adaptation in a flagellate protozoan
Marvin Cantor	Metabolic adaptation in a flagellate protozoan
David C. Grant	Specific diversity in an intertidal community
Copeland MacClintock	Microstructure of the shell in Gastropoda
Melbourne R. Carriker	An aerial overview of the major marine habitats of the Cape Cod region
Jack B. Pearce	A pilgrimage to Ellerslie (P.E.I., Canada) and its surrounding benthic communities
Donald F. Squires	Fossil coral thickets
Robert H. Parker	The 1958–59 Downwind Expedition to Easter Island
Thomas J. M. Schopf	Conodonts of the Trenton Group (Ordovician) in New York and Southern Ontario, Canada
Kay W. Petersen	On the origin of Metazoa
Robert R. Hessler	*Derocheilocaris typicus* revisited
Daryl Sweeney	What good is dopamine; clams; biochemical evolution?

THE LABORATORY STAFF

Homer P. Smith, General Manager

Miss Jane Fessenden, Acting Librarian
Carl O. Schweidenback, Manager, Supply Department
Irvine L. Broadbent, Office Manager
Robert Kahler, Superintendent, Buildings and Grounds
Robert B. Mills, Manager, Department of Research Service

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Robert Gunning
Donald B. Lehy
Ralph H. Lewis
Russell F. Lewis
Henry F. Potter
Robert H. Walker, Jr.

DEPARTMENT OF RESEARCH SERVICE

Gail M. Cavanaugh
Lowell V. Martin
Miss Margaret E. Scott
Frank E. Sylvia

SUPPLY DEPARTMENT

Arno J. Bowden
David H. Graham
Mrs. E. Green
Robert W. Hampton
Robert O. Lehy
Paul Shave
Bruno F. Trapasso
John J. Valois
Hallett S. Wagstaff

DINING HALL AND HOUSING

Robert T. Martin, Manager, Food Service
Mrs. Elizabeth Kuil, Supervisor, Dining Room
Mrs. Ellen T. Nickelson, Supervisor, Dormitories
Alan G. Lunn, Supervisor, Cottage Colony

3. Investigators: Lalor, Lillie and Grass Fellows; Students

Independent and Beginning Investigators, 1964

Abbott, Bernard C., Professor of Physiology and Biophysics, University of Illinois
Adelberg, Edward A., Professor and Chairman, Department of Microbiology, Yale University
Adelman, William J., Jr., Associate Professor of Physiology, University of Maryland
Allen, Robert Day, Associate Professor of Biology, Princeton University
Anderson, Everett, Professor of Zoology, University of Massachusetts
Armstrong, Philip B., Chairman, Department of Anatomy, State University of New York, College of Medicine at Syracuse
Arnold, John M., Lerner Marine Laboratory of the American Museum of Natural History
Auclair, Walter, Assistant Professor of Zoology, University of Cincinnati
Austin, C. R., Member of External Scientific Staff, Medical Research Council of Great Britan
Baker, Robert F., Radiation Physicist, Brown University
Bang, Frederik B., Professor of Pathobiology, The Johns Hopkins University
Bardach, John E., Professor of Zoology, University of Michigan
Barlow, Robert B., The Rockefeller Institute
Barth, L. G., Professor of Zoology, Columbia University
Barth, L. J., Professor of Zoology, Barnard College
Bauer, G. Eric, Profesor of Anatomy, University of Minnesota
Baumann, Fritz, National Institute of Neurological Diseases and Blindness, National Institutes of Health
Baylor, Martha Barnes, Marine Biological Laboratory
Beatty, Richard Alan, Senior Principal Scientific Officer, Agricultural Research Council Unit of Animal Genetics, Great Britain
Belamarich, Frank A., Assistant Professor of Biology, Boston University
Bell, Eugene, Associate Professor of Biology, Massachusetts Institute of Technology
Bennett, M. V. L., Associate Professor of Neurology, Columbia University, College of Physicians and Surgeons
Biggers, John D., Professor of Reproductive Physiology, University of Pennsylvania
Billiar, Reinhart B., Research Fellow, Harvard Medical School
Binstock, Leonard, Electronic Engineer (Instrumentation), National Institutes of Health
Boolootian, Richard A., Associate Professor of Zoology, University of California, Los Angeles
Brandt, Phillip W., Assistant Professor of Anatomy, Columbia University
Branham, Joseph M., Assistant Professor of Biology, Oglethorpe University

Brinley, F. J., Assistant Professor of Physiology, The Johns Hopkins School of Medicine
Brown, Donald D., Staff Member in Biochemistry, Carnegie Institution of Washington
Brown, Frank A., Jr., Morrison Professor of Biology, Northwestern University
Bryant, S. H., Associate Professor of Pharmacology, University of Cincinnati
Brzin, Miro, Senior Research Associate, University of Ljubljana
Burch, Helen B., Associate Professor of Pharmacology, Washington University
Burnett, Allison L., Associate Professor of Biology, Western Reserve University
Cantor, Marvin H., Postdoctoral Fellow, Systematics-Ecology Program, Marine Biological Laboratory
Carlson, Francis D., Chairman and Professor of Biophysics, The Johns Hopkins University
Carriker, Melbourne R., Director, Systematics-Ecology Program, National Institutes of Health
Carter, John, Postdoctoral Fellow, Systematics-Ecology Program, Ford Foundation
Cheney, Ralph Holt, Professor of Biology, Brooklyn College, The City University of New York
Child, Frank M., Assistant Professor of Zoology, University of Chicago
Claff, C. Lloyd, Treasurer and Chief Investigator, Single Cell Research Foundation, Inc.
Clark, Eloise E., Assistant Professor of Zoology, Columbia University
Clement, A. C., Professor of Biology, Emory University
Cole, Kenneth S., Chief, Laboratory of Biophysics, NINDB, National Institutes of Health
Colwin, Arthur L., Professor of Biology, Queens College, The City University of New York
Colwin, Laura Hunter, Lecturer in Biology, Queens College, The City University of New York
Cook, Philip William, Assistant Professor of Botany, University of Vermont
Cooperstein, Sherwin J., Associate Professor of Anatomy, Assistant Dean, Medical School, Western Reserve University
Copeland, Eugene, Chairman and Professor of Zoology, Tulane University
Costello, Donald Paul, Kenan Professor of Zoology, University of North Carolina
Crowell, Sears, Professor of Zoology, Indiana University
DeHaan, Robert L., Research Embryologist, Carnegie Institution of Washington
de Lorenzo, A. J., Director, Anatomical and Pathological Research Laboratories, The Johns Hopkins University School of Medicine
Dettbarn, Wolf-Dietrich, Assistant Professor of Neurology, Columbia University, College of Physicians and Surgeons
de Villafranca, George W., Professor of Zoology, Smith College
Dische, Zacharias, Professor of Biochemistry, Columbia University, College of Physicians and Surgeons
Dunham, Philip B., Assistant Profesor of Zoology, Syracuse University
Ebert, James D., Director, Department of Embryology, Carnegie Institution of Washington
Eckert, Roger, Assistant Professor of Zoology, Syracuse University
Edds, Mac V., Jr., Chairman and Professor of Medical Science, Brown University
Ehrenstein, Gerald, Physicist, National Institutes of Health
Eskridge, Rosemary Waite, Special Research Assistant of Biochemistry, State University of New York at Buffalo
Evoy, William H., Grass Fellow, University of Oregon
Failla, Patricia McClement, Associate Biophysicist, Argonne National Laboratory
Farmanfarmaian, Allahverdi, Professor of General Physiology, Pahlavi University, Shiraz, Iran
Fisher, Frank M., Jr., Assistant Professor of Biology, Rice University
Fishman, Louis, Assistant Research Professor, New York University College of Dentistry
Freeman, Alan R., Postdoctoral Trainee Fellow USPHS, Columbia University
Frumento, Antonio S., Postdoctoral Fellow, University of Maryland
Fujio, Hajime, Department of Microbiology, University of Pennsylvania Medical School
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Furshpan, Edwin J., Assistant Professor of Neurophysiology, Harvard Medical School
Garcia, Horacio A., Grass Fellow, Columbia University
Geilenkirchen, W. L. M., Lecturer, University of Utrecht, Holland

Gelfant, Seymour, Associate Professor of Zoology, Syracuse University
German, James L., III, Director and Associate Professor of Division of Human Genetics, Cornell University Medical School
Gibbons, I. R., Assistant Professor, Biological Laboratories, Harvard University
Gilbert, Daniel L., Physiologist, National Institutes of Health
Gimenez, Maximo, Grass Fellow, Columbia University, College of Physicians and Surgeons
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Goldman, Lawrence, Postdoctoral Fellow, Columbia University, College of Physicians and Surgeons
Grant, David, Research Associate, Systematics-Ecology Program
Grant, Philip, Program Director, Developmental Biology, National Science Foundation
Green, Jonathan, Postdoctoral Research Fellow, The Johns Hopkins University
Grey, Howard, The Rockefeller Institute
Grosch, Daniel S., Professor of Genetics, North Carolina State of the University of North Carolina at Raleigh
Gross, Paul R., Associate Professor of Biology, Brown University
Grundfest, Harry, Professor of Neurology, Columbia University, College of Physicians and Surgeons
Guttman, Rita, Associate Professor of Biology, Brooklyn College
Hadorn, E., Director, Zoologisch-Vergl. Anatomisches Institut, Der Universität Zurich, Switzerland
Hagins, William A., NIAMD, National Institutes of Health
Halvorson, H. O., Professor of Bacteriology, University of Wisconsin
Harding, Clifford V., Associate Professor of Physiology, Columbia University, College of Physicians and Surgeons
Harosi, Ferenc, Electronics Engineer, The Rockefeller Institute
Hastings, J. Woodland, Professor of Biochemistry, University of Illinois
Hayashi, Teru, Chairman and Professor of Zoology, Columbia University
Hegyeli, Andrew, Institute for Muscle Research, Marine Biological Laboratory
Herndon, Walter R., Head and Professor of Botany, University of Tennessee
Hervey, John P., Senior Electronics Engineer, The Rockefeller Institute
Higashino, Shoji, Research Associate in Physiology, Gunma University, Japan and Columbia University
Higgins, Don Cheney, Assistant Professor of Internal Medicine, Yale University
Hiramoto, Yukio, Assistant Professor at Misaki Marine Biological Station, University of Tokyo
Holz, George G., Chairman and Professor of Microbiology, State University of New York, Upstate Medical Center
Hope, William Duane, Associate Curator, Division of Marine Invertebrates, United States National Museum
Hoskin, Francis C. G., Assistant Professor of Neurology, Columbia University, College of Physicians and Surgeons
Humphreys, Tom, Assistant Professor of Biology, Massachusetts Institute of Technology
Hunter, W. D. Russell, Professor of Zoology, Syracuse University
Huver, Charles W., Assistant Professor of Anatomy, University of Illinois
Inoué, Shinya, Chairman and Professor of Cytology, Dartmouth Medical School
Isenberg, Irvin, Institute for Muscle Research, Marine Biological Laboratory
Janoff, Aaron, Assistant Professor of Pathology, New York University School of Medicine
Johnson, Frank H., Professor of Biology, Princeton University
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Kane, Robert E., Assistant Professor of Cytology, Dartmouth Medical School
Karlin, Arthur, Research Associate, Columbia University, College of Physicians and Surgeons
Karasaki, Shuichi, Biology Division, Oak Ridge National Laboratory
Karush, Fred, Professor of Microbiology, University of Pennsylvania Medical School
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Kaley, Gabor, Assistant Professor of Pathology, New York University

Kempton, Rudolf T., Professor of Zoology, Vassar College
Keosian, John, Professor of Biology, Rutgers The State University
Kessel, Richard G., Assistant Professor of Zoology, State University of Iowa
King, Thomas J., Head, Department of Embryology, The Institute for Cancer Research
Kinne, Otto, Director and Professor of Biologische Anstalt Helgoland, Hamburg-Altona, Palmaille, 9, Germany
Kleinholz, Lewis H., Professor of Biology, Reed College
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Lansing, Albert, Professor of Anatomy, University of Pittsburgh
Lash, James W., Assistant Professor of Anatomy, University of Pennsylvania Medical School
Laster, Leonard, Chief of Gastroenterology Unit, National Institute of Arthritis and Metabolic Diseases
Lazarow, Arnold, Head and Professor of Anatomy, University of Minnesota
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Lerman, Sidney, Associate Professor of Ophthalmology & Assistant Professor of Biochemistry, University of Rochester School of Medicine & Dentistry
Levine, Lawrence, Professor of Biochemistry, Brandeis University
Levy, Milton, Chairman and Professor of Biochemsitry, New York University College of Dentistry
Liebman, Paul A., Assistant Professor of Physiology, University of Pennsylvania
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Lopez, Enrique, Research Associate in Neurology, Columbia University, College of Physicians and Surgeons
Lorand, L., Professor of Chemistry, Northwestern University
Love, Warner E., Associate Professor of Biophysics, The Johns Hopkins University
MacNichol, Edward F., Professor of Biophysics, The Johns Hopkins University
Maggio, Rachele, Assistant Professor, Institute of Comparative Anatomy, University of Palermo, Italy
Mahler, Henry R., Professor of Chemistry, Indiana University
Malkin, Leonard I., Research Associate of Biology, Brown University
Marchalonis, John, The Rockefeller Institute
Marmasse, Claude, The Commonwealth Fund, Marine Biological Laboratory
Marsland, Douglas, Research Professor, New York University
McElroy, W. D., Professor of Biology, The Johns Hopkins University
Mellon, DeForest, Jr., Assistant Professor of Biology, University of Virginia
Mendelson, Martin, Professor in Physiology, New York University
Metz, Charles B., Professor of Biology in the Institute for Space Biosciences, Florida State University
Miledi, Ricardo, Reader in Biophysics, University College of London, England
Milkman, Roger Dawson, Associate Professor of Zoology, Syracuse University
Miller, Faith S., Assistant Professor of Anatomy, Tulane University
Miller, James A., Professor and Chairman of Anatomy, Tulane University
Mohler, J. D., Associate Professor of Zoology, Oregon State University
Mohri, Hideo, Research Associate of Biology, University of Tokyo
Monroy, Alberto, Professor of Comparative Anatomy, University of Palermo, Italy
Moore, John W., Associate Professor of Physiology, Chief, Laboratory of Cellular Neurophysiology, Duke University
Moscona, A. A., Professor of Zoology, University of Chicago
Mullins, L. J., Professor of Biophysics, University of Maryland
Nace, Paul Foley, Professor, Research Unit in Molecular Biology, McMaster University, Canada
Naqai, Reiko, Research Associate of Biology, Princeton University
Nakamura, Yutaka, Research Fellow, Columbia University, College of Physicians and Surgeons

Nasatir, Maimon, Assistant Professor and Assistant to the Dean, Pembroke College
Nelson, Leonard, Associate Professor of Physiology, Emory University
Nichols, H. Wayne, Assistant Professor of Botany, Washington University at St. Louis
Nyborg, Wesley L., Professor of Physics, University of Vermont
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Parpart, Arthur K., Chairman and Professor of Biology, Princeton University
Pearce, John Bodell, Research Associate, Systematics-Ecology Program, National Science Foundation
Person, Philip, Chief, Special Dental Research Laboratory, VA Hospital, Brooklyn
Porter, Keith R., Head and Professor of Biological Laboratories, Harvard University
Rabin, Harvey, Assistant Professor of Pathology, The Johns Hopkins University
Rasmussen, Rosemary Crockett, Research Associate, State University of New York, Upstate Medical Center
Read, Clark P., Professor of Biology, Rice University
Rebhun, Lionel I., Associate Professor of Biology, Princeton University
Reporter, Minocher, Carnegie Institution of Washington
Reuben, John P., Assistant Professor of Neurology, Columbia University, College of Physicians and Surgeons
Rice, Robert V., Senior Fellow, Mellon Institute
Rose, S. Meryl, Professor of Experimental Embryology, Tulane University
Rosenberg, Philip, Assistant Professor in Neurology, Columbia University, College of Physicians and Surgeons
Rosenkranz, Herbert S., Assistant Professor of Microbiology, Columbia University, College of Physicians and Surgeons
Roslansky, John D., Institute for Muscle Research, Marine Biological Laboratory
Roth, Jay S., Professor of Biochemistry, University of Connecticut
Rugh, Roberts, Associate Professor of Radiology (Biology), Columbia University
Rustad, Ronald C., Associate Professor of Biology, Western Reserve University
Sanders, Howard L., Research Associate in Ecology, Woods Hole Oceanographic Institution
Sato, Hidemi, Assistant Professor of Cytology, Dartmouth Medical School
Saunders, John W., Chairman and Professor of Biology, Marquette University
Schneiderman, Howard A., Chairman and Professor of Biology, Western Reserve University
Schmeer, Sister M. Rosarii, Co-Director of Research, St. Mary of the Springs
Schnitzler, Ronald Michael, Research Fellow, University of Vermont
Schopf, Thomas J. M., Postdoctoral, Systematics-Ecology Program, Ford Foundation
Schuel, Herbert, Postdoctoral Fellow, Northwestern University
Scott, Allan C., Professor of Biology, Colby College
Scott, Sister Florence Marie, Chairman and Professor of Biology, Seton Hill College
Scott, George T., Chairman and Professor of Biology, Oberlin College
Senft, Joseph P., USPHS Postdoctoral Fellow, University of Maryland
Shaw, Charis, Research Associate, Tulane University
Shemin, David, Professor of Biochemistry, Columbia University
Sherman, Irwin W., Assistant Professor of Zoology, University of California, at Riverside
Sichel, F. J., Chairman and Professor of Physiology and Biophysics, College of Medicine, University of Vermont
Simon, Joseph L., Sandeen Memorial Fellow, University of Florida
Simpson, Sidney B., Jr., Postdoctoral Fellow, Department of Anatomy, Western Reserve Medical School
Singer, Irwin, Research Associate, National Institute of Mental Health
Sjodin, Raymond A., Associate Professor of Biophysics, University of Maryland
Slater, Clarke Rothwell, Grass Fellow, University College, London, England
Slobodkin, Lawrence B., Professor of Zoology, University of Michigan
Smelser, George K., Professor of Anatomy, Columbia University
Smith, Edmund H., National Institutes of Health Fellow, Systematics-Ecology Program

Smith, Thomas Graves, Research Neurophysiologist, NINDB, National Institutes of Health
Smith, William Roy, School of Hygiene & Public Health, The Johns Hopkins University
Speidel, Carl C., Professor of Anatomy, University of Virginia
Spindel, William, Professor of Chemistry, Rutgers University
Spirtes, M. A., Associate Professor of Pharmacology, Hahnemann Medical College
Steinbach, H. Burr, Chairman and Professor of Zoology, University of Chicago
Strittmatter, Philipp, Associate Professor of Biochemistry, Washington University
Stunkard, Horace W., Research Associate, American Museum of Natural History
Surgenor, Douglas M., Dean of Medical School, Chairman of Biochemistry, State University of New York at Buffalo
Szabó, George, Assistant Professor of Anatomy, Harvard Medical School
Szent-Györgyi, Albert, Director and Chief Investigator, Institute for Muscle Research, Marine Biological Laboratory
Takata, Mitsuru, Assistant Professor of Physiology, Duke University
Takenaka, Toshifumi, National Institutes of Health
Tasaki, Ichiji, Acting Chief, Laboratory of Neurobiology, NIMH, National Institutes of Health
Taylor, Peter, Postdoctoral Fellow, Systematics-Ecology Program, National Science Foundation
Taylor, Robert E., Associate Chief, Biophysics Laboratory, NINDB, National Institutes of Health
Taylor, Walter Rowland, Assistant Professor of Oceanography, The Johns Hopkins University
Tencer, Renée, Assistant, University of Brussels, Brussels
Tilney, Lewis G., Postdoctoral Fellow, Harvard University
Torch, Reuben, Associate Professor of Zoology, University of Vermont
Travis, David M., Assistant Professor of Pharmacology & Therapeutics, University of Florida College of Medicine
Trinkaus, J. P., Professor of Biology, Yale University
Troll, Walter, Associate Professor, New York University Medical Center, Institute of Industrial Medicine
Tweedell, Kenyon S., Associate Professor of Biology, University of Notre Dame
Usherwood, Peter N. R., Research Associate, Columbia University, College of Physicians and Surgeons
Van Holde, K. E., Associate Professor of Chemistry, University of Illinois
Van Vunakis, Helen, Associate Professor of Biochemistry, Brandeis University
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Wallace, Robin A., Research Associate, Biology Division, Oak Ridge National Laboratory
Walsh, Gerald Edward, Postdoctoral Research Associate, Systematics-Ecology Program, National Science Foundation
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Webb, H. Marguerite, Associate Professor, Research Assistant, Goucher College and Northwestern University
Weiss, Leon, Associate Professor of Anatomy, The Johns Hopkins University School of Medicine
Wichterman, Ralph, Professor of Biology, Temple University
Wilce, R. T., Assistant Professor of Botany, University of Massachusetts
Wilson, Walter L., Associate Professor of Physiology and Biophysics, University of Vermont College of Medicine
Winters, Robert W., Professor of Pediatrics & Career Scientist, Columbia University, College of Physicians and Surgeons
Wyttenbach, Charles R., Assistant Professor of Anatomy, University of Chicago
Zigman, S., Instructor in Biochemistry, University of Rochester
Zimmerman, Arthur M., Assistant Professor of Pharmacology, State University of New York, Downstate Medical Center
Zullo, Victor A., Assistant Director, Resident Systematist, Systematics-Ecology Program, Ford Foundation

Lalor Fellows, 1964

Richard Alan Beatty, Senior Fellow, Agricultural Research Council Unit of Animal Genetics, United Kingdom
Joseph M. Branham, Oglethorpe University
Yukio Hiramoto, Misaki Marine Biological Station, Japan
Charles W. Huver, University of Illinois
Rachele Maggio, University of Palermo, Italy
Hideo Mohri, University of Tokyo, Japan
Robert W. Winters, Columbia University, College of Physicians and Surgeons

Lillie Fellow, 1964

E. Hadorn, Der Universität Zurich, Switzerland

Grass Fellows, 1964

Neil Davidson, State University of New York, Downstate Medical Center, Brooklyn
William H. Evoy, University of Oregon
Horacio A. Garcia, Columbia University, College of Physicians and Surgeons
Maximo Gimenez, Columbia University, College of Physicians and Surgeons
Ricardo Miledi, Forbes Lecturer, University College, London
Clarke Rothwell Slater, University College, London

Research Assistants, 1964

Acquaviva, Patricia Ann, Seton Hill College
Altshuler, Bernard, New York University Medical Center
Anonellis, Blenda C., Western Reserve University
Apicella, James V., University of Pittsburgh
Ardwin, Lindsay, S., Columbia University
Armstrong, Judy, Western Reserve University
Aronson, Wendy S., Vassar College
Ashworth, John Michael, Leicester University, England
Baird, Spencer L., Institute for Muscle Research
Barnhill, Robert, Capitol Radio Engineering Institute
Berrien, Judi, Princeton University
Bikle, Daniel, Harvard University
Blair, Marion H., McMaster University, Canada
Blumenthal, Daniel S., Oberlin College
Bollinger, M. Susan, University of Maryland
Brady, Francine, Syracuse University
Brever, Anthony Carl, Princeton University
Brungard, Joanne, Harvard Medical School
Burger, Richard, Princeton University
Carden, George Alexander, III, Columbia University, College of Physicians and Surgeons
Chaffee, Richard B., Jr., Syracuse University
Chagnon, Suzanne, University of Vermont
Chany, Amos Hwei-Cheh, Columbia University
Chasis, Joel Anne, New York University School of Medicine
Crouse, Frances W., Biologische Anstalt Helgoland
Daniels, Charles, Duke University
Davidson, Neil, State University of New York
De Luca, Marlene, The Johns Hopkins University
Dimino, Patricia, Columbia University
Doane, Marshall G., University of Maryland School of Medicine
Dumont, James N., University of Massachusetts
Eisenberg, Henry W., Columbia University
Fedoroff, Nina, Syracuse University

Fisher, Ellen D. B., Columbia University
Fitzjarrell, Austin T., Tulane University
Foran, Elizabeth H., Smith College
Freeman, Susan G., Columbia University
Fu, Karen, Columbia University, College of Physicians and Surgeons
Galton, Virginia, Harvard Medical School
Gebelein, Conrad Dennis, The Johns Hopkins University
Gedmintas, Dana, University of Chicago
Gottdiener, Donna, Vassar College
Gramss, Elise, Institute for Muscle Research
Harris, Edward M., Duke University
Harvey, Jenette, Johns Hopkins School of Medicine
Hechter, Michael, Columbia University
Heckman, James D., Princeton University
Hegab, El-Sayed, Tulane University
Hitchcock, Susan M., Columbia University, College of Physicians and Surgeons
Hodes, Barton L., Jefferson Medical College
Jaffee, Stephen, New York University School of Medicine
Johnson, Kurt E., The Johns Hopkins University
Kaufman, Robert G., Columbia University
Kehlenbeck, Edna, Syracuse University
Kilejian, Araxie, Rice University
Kimball, Frances, Reed College
Kirschberg, Gordon J., Columbia University, College of Physicians and Surgeons
Larsen, William J., Wesleyan University
Lester, Gordon James, University of Minnesota
Levine, Marilyn, Western Reserve University
MacNamara, Gael R., Columbia University, College of Physicians and Surgeons
Mazia, Judith Ann, University of Chicago
McGilvray, Jean Marie, Dartmouth Medical School
McEnaney, Barbara, Marquette University
Meismer, Donald M., University of Cincinnati
Miller, Sandra M., University of Maryland
Mittenthal, Jay E., The Johns Hopkins University
Mohl, Robert L., Hahnemann Medical College
Mosser, Jerry L., Indiana University and The Rockefeller Institute
Munday, John C., University of Illinois
Munro, George F., University of Rochester
Munro, Judith L., University of Rochester
Murphy, Anne M., University of Maryland
Newman, Brooke, Institute for Muscle Research
Olmsted, Charles E., University of Chicago
Paimre, Marve, State University of New York, Downstate Medical Center
Panny, Susan R., Columbia University
Pawelek, John M., Brown University
Powers, Earl G., University of Cincinnati
Rasmussen, Leif, Carlsberg Foundation
Ravitz, Melvyn J., University of Vermont
Ray, Patricia, Seton Hill College
Reale, Vincent F., Princeton University
Richmond, Arthur P., Single Cell Research Foundation, Inc.
Robertson, C. W., American Museum of Natural History
Rosenbluth, Raja, Institute for Muscle Research
Sander, Greta, Princeton University
Schachter, Meri, Columbia University
Setlow, Peter, Brandeis University
Sindelar, William, Western Reserve University
Sloane, Eleanore M., Mellon Institute

SLOANE, MOLLA R., Wellesley College
THOMAS, JUNE M., University of California, Los Angeles
TOBEY, JOHN H., Maine Vocational Technical Institute
TRAVIS, JEANNE D., University of Florida
TRENHAFT, PAUL STEVEN, Oberlin College
TSUKIDATE, JIUNICHI, Haskins Laboratories
TUCKER, ROBERT W., Dartmouth Medical School
TURNER, VILIA G., University of California, Los Angeles
TUTUNJIAN, JEAN, Columbia University, College of Physicians and Surgeons
UEHARA, MARGARET H., University of Hawaii
VAN PRAAG, DINA, New York University
VASQUEZ, CARMEN, University of Michigan
WALDBAUM, MARK, Hahnemann Medical College
WASSERMAN, ELEANOR, Brandeis University
WEINER, BEVERLY, Harvard University
WILSON, LOUISE P., Wellesley College
YUYAMA, SHUHEI, Western Reserve University
ZOLLINGER, WILLIAM K., JR., University of Pittsburgh Medical School

Library Readers, 1964

ATWOOD, KIMBALL C., Professor of Microbiology, University of Illinois
BALL, ERIC G., Professor of Biological Chemistry, Harvard Medical School
BERNE, ROBERT M., Professor of Physiology, Western Reserve University
BRIDGMAN, ANNA JOSEPHINE, Chairman and Professor of Biology, Agnes Scott College
BUTLER, ELMER G., Osborn Professor of Biology, Princeton University
CARBON, JOHN A., Research Associate, Department of Biochemistry, Abbott Laboratories
CHASE, AURIN M., Professor of Biology, Princeton University
CLARK, ARNOLD M., Professor of Biology, University of Delaware
COHEN, SEYMOUR S., Chairman, Department of Therapeutic Research, University of Pennsylvania School of Medicine
DAVIS, BERNARD D., Head, Department of Bacteriology and Immunology, Harvard Medical School
EDER, HOWARD A., Professor of Medicine, Albert Einstein College of Medicine
GABRIEL, MORDECAI L., Professor of Biology, Brooklyn College
GINSBERG, HAROLD S., Chairman, Department of Microbiology, University of Pennsylvania School of Medicine
GREEN, JAMES W., Professor of Physiology, Rutgers University
HANDLER, PHILIP, Professor of Biochemistry, Duke University
HESSLER, ANITA YOUNG, Marine Biological Laboratory
HODES, ROBERT, Research Associate, Department of Neurophysiology, The Mount Sinai Hospital
ISSELBACKER, KURT J., Chief, Gastrointestinal Unit, Massachusetts General Hospital and Assistant Professor of Medicine, Harvard Medical School
JACOBS, M. H., Emeritus Professor of General Physiology, University of Pennsylvania
KALTENBACH, JANE COUFFER, Assistant Professor of Zoology, Mount Holyoke College
KASHA, MICHAEL, Director, Institute of Molecular Biophysics, Florida State University
KLEIN, MORTON, Professor of Immunology, Temple University Medical School
LEIGHTON, JOSEPH, Professor of Pathology, University of Pennsylvania School of Medicine
LEVINE, RACHMIEL, Chairman, Department of Medicine, New York Medical School
LEVINTHAL, CYRUS, Professor of Biophysics, Massachusetts Institute of Technology
LINEAWEAVER, THOMAS H., III, Marine Biological Laboratory
MARKS, PAUL A., Associate Professor of Medicine, Columbia University, College of Physicians and Surgeons
MATEYKO, GLADYS MARY, Associate Professor of Biology, Washington Square College, New York University
MEYER, KARL, Professor of Biochemistry, Columbia University
MOULTON, JAMES M., Associate Professor of Biology, Bowdoin College

Nason, Alvin, Professor of Biology, Associate Director, McCollum-Pratt Institute, The Johns Hopkins University
Needleman, Saul B., Senior Research Biochemist, Abbott Laboratories
Novikoff, Alex B., Research Professor, Albert Einstein College of Medicine
Nowotny, Alois H., Professor of Immunochemistry, Temple University School of Medicine
Overton, Jane H., Associate Professorial Lecturer in Biology, University of Chicago
Rapport, Maurice M., Professor of Biochemistry, Albert Einstein College of Medicine
Rowland, Lewis P., Associate Professor of Neurology, Columbia University, College of Physicians and Surgeons
Russell, Henry D., Museum of Comparative Zoology, Harvard University
Spiegel, Melvin, Associate Professor of Biology, Dartmouth College
Sprague James M., Professor of Anatomy, University of Pennsylvania
Stetten, Marjorie R., Research Professor, Rutgers Medical School
Sudak, Frederick N., Assistant Professor of Physiology, Albert Einstein College of Medicine
Swanson, Carl P., William D. Gill Professor of Biology, The Johns Hopkins University
Szent-Györgyi, Andrew G., Professor of Biophysics, Dartmouth Medical School
Wainio, Walter, Professor of Biochemistry, Rutgers The State University of New Jersey
Warner, Robert C., Professor of Biochemistry, New York University School of Medicine
Wheeler, George E., Associate Professor of Biology, Brooklyn College
Wilson, Thomas Hastings, Associate Professor of Physiology, Harvard Medical School
Yntema, Chester L., Professor of Anatomy, State University of New York, Upstate Medical Center
Zacks, Sumner I., Assistant Professor of Neuropathology, Pennsylvania Hospital, University of Pennsylvania
Zorzoli, Anita, Chairman, Professor of Physiology, Vassar College

Students, 1964

All students listed completed the formal course program, June 17–July 27. Asterisk indicates students completing Post-Course Research Program, July 28–August 31.

ECOLOGY

*Adamson, Jean M., Allegheny College
Allessio, Mary L., University of Colorado
Avery, Patricia P., Wheaton College
*Barvenik, Frank W., University of Connecticut
*Buchsbaum, Vicki M., Stanford University
*Calder, William Alexander, Jr., Duke University
Gjessing, Helen Witton, College of the Virgin Islands
*Heatfield, Barry Mark, University of California
Jones, Meredith Howe, Boston University
Jones, Nancy Gale, Oberlin College
Koetzer, Kenneth L., University of Rhode Island
*Lloyd, Margaret C., Bryn Mawr College
Mayo, Charles A., III, Dartmouth College
Quinn, Sister Genevieve, Catholic University of America
*Rea, Ina K., Indiana University
*Richardson, W. Norman, Earlham College
White, Joseph James, University of Illinois

EMBRYOLOGY

Baril, Earl Francis, University of Connecticut
Berrill, Michael, McGill University
*Connell, Carolyn, Indiana University
*Dick, Miriam, Brandeis University

*Gaede, LeRoy Lewis, Rensselaer Polytechnic Institute
*Goldizen, Vernon Claire, Western Reserve University
*Gould, Meredith C., Stanford University
Hayashi, Masaki, University of Illinois
*Heidger, Paul McClay, Jr., Tulane University
*Held, William Allen, Marquette University
Inselburg, Joseph, Brandeis University
Kaplan, Stanley, University of Miami
*Kopp, Lowell Ellis, Massachusetts Institute of Technology
*Larsen, Lyndell Louise, The Rockefeller Institute
Mortensen, Richard, Purdue University
*Percus, Jerome Kenneth, New York University
Pringle, John Robert, Harvard University
*Read, Margaret Tyler, Harvard University
*Reigart, John Routt, II, Dartmouth Medical School
Rogers, Mary Elizabeth, Yale University

BOTANY

Burg, Carol Ann, University of Connecticut
*Bytnar, Patricia Ann, Seton Hill College
Conner, Clarice Marie, University of Wisconsin
*Holt, Buford Reid, University of Tennessee
*Howell, Stephen H., The Johns Hopkins University
Kevin, Sister M. Petra, Fordham University
Kies, Robert Ludwig, University of Erlangen, Germany
Kochert, Gary Dean, Indiana University
*Lee, Thomas F., Clark University
McLean, Robert J., University of Connecticut
*Prince, Jeffrey S., University of Massachusetts
*Ramus, Joseph Stephen, University of California, Berkeley
Smith, Joyce Eileen, Cornell University
Strother, John Lance, Washington University, St. Louis
*Trerice, Elizabeth Mabel, Dalhousie University, Halifax, Nova Scotia
*Urban, Paul, Tufts University
Waer, Richard Dennis, University of Arizona
*Weber, Jill Louise, Vassar College
*Wilcox, Robert Stimson, University of Michigan

PHYSIOLOGY

*Barbour, Stephen David, Temple University
*Biber, Michael Peter, University of Chicago Medical School
*Carolan, Robert Mills, Dartmouth Medical School
*Craig, Nessly Coile, University of Pennsylvania
*Converse, Carolyn Ann, Brown University
*Elfbaum, Stanley Goodman, Northwestern University
*Etzler, Marilynn Edith, Washington University, St. Louis
Gazith, Joseph, Vanderbilt University
Giberman, Eldad, Massachusetts Institute of Technology
*Gold, Lawrence Marshall, University of Connecticut
*Hatling, Donna Lynne, Columbia University
*Hauschka, Peter Voorhess, Amherst College
Haytler, Peter G., E. I. duPont de Nemours & Company
*Juras, Danute, Marquette University
*Kubai, Donna Farolino, University of Wisconsin
*Lattmann, Eaton Edward, The Johns Hopkins University

*Lloyd, David Albert, University of Illinois
*Mandel, Morton, Stanford University School of Medicine
*Nicholson, Anne, University of Pennsylvania School of Medicine
deRazumney, Celia Ester Freda, University of Pennsylvania School of Medicine
Rothenstein, Arthur Stanley, Rutgers, The State University
*Sparks, Harvey V., Harvard Medical School
Switzer, Sam, Albert Einstein College of Medicine
*Terando, Sister Mary Loretta, Saint Louis University
*Terrell, Kathleen Lois, Columbia University, College of Physicians and Surgeons
*Ward, John Clive, The Johns Hopkins University
*Ward, Raymond Leland, University of California, Livermore
*Wechsler, James Alan, Yale University
*Weinberg, Eric S., The Rockefeller Institute
*White, Eric S., Dartmouth Medical School

INVERTEBRATE ZOOLOGY

Allen, Jeffrey Charles, Oberlin College
Applebaum, Richard, Columbia University
Bartizal, Frederick Joseph, Beloit College
Bennett, Judith Ann, Syracuse University
Bolender, Robert Paul, Columbia University
Boyd, Carl M., Dalhousie University, Halifax, Nova Scotia
Chane, Paula Frances, Tulane University
Coggeshall, Richard Edwin, Harvard Medical School
Cotman, Carl Wayne, Wesleyan University
Dennaker, Germaine Suzanne, Morgan State College
Fischer, Barbara Ann, St. Louis University
Hall, Barbara Sue, College of St. Mary of the Springs
Hine, Charles Risk, Lafayette College
Hunter, William Bruce, University of California, Santa Barbara
Jampol, Lee Merrill, Yale University
Johnson, Kurt Edward, The Johns Hopkins University
Kaufman, Robert Gordon, Columbia University
Koering, Marilyn J., University of Wisconsin
Koo, Helen Ping-Ching, University of Minnesota
Langreth, Susan Grant, University of Chicago
Meadows, Robert T., Syracuse University
Nollen, Paul Marion, Purdue University
Nutt, John Gordon, Jr., Rice University
Page, Charles Henry, Yale University
Pawalek, John Mason, Brown University
Pettit, Barbara, Marquette University
Reuss, Cecilia Monica, Marquette University
Robinson, Carolyn Anne, Clark University
Rundles, Charlotte, Duke University
Stine, Deborah Clare, Wilson College
Toth, Steven Edward, Bowling Green State University
Tracy, Susan Frances, University of Massachusetts
Walcott, Benjamin, University of Oregon
Waldron, Ingrid Lore, University of California, Berkeley
Walter, Mary A., Ripon College
Walters, David Royal, Harvard University
Ward, Oscar Gardien, Jr., Purdue University
Werner, Betsey Ann, Drew University
Whisnant, Betty Lynn, Duke University
Zeimen, Sister Maria Goretti, Catholic University of America

4. FELLOWSHIPS AND SCHOLARSHIPS, 1964

Lucretia Crocker Scholarship:
VICKI M. BUCHSBAUM, Ecology Course
BUFORD R. HOLT, Botany Course

Edwin Linton Memorial Endowment of the Washington and Jefferson College:
PATRICIA ANN BYTNAR, Botany Course

Turtox Scholarship Fund:
JOHN BUSHNELL

5. TRAINING PROGRAMS

FERTILIZATION AND GAMETE PHYSIOLOGY TRAINING PROGRAM

I. INSTRUCTORS

C. B. METZ	Florida State University, in charge of program
C. R. AUSTIN	Cambridge University, England
JOHN BIGGERS	University of Pennsylvania
ALBERTO MONROY	University of Palermo, Italy
LEONARD NELSON	Emory University

II. ASSISTANTS

RACHELE MAGGIO	University of Palermo, Italy

III. STUDENTS

R. BERKELEY	University of Pennsylvania
J. F. FALLON	Marquette University
L. E. FRANKLIN	Florida State University
M. S. GOROVSKY	University of Chicago
R. HALLBERG	The Johns Hopkins University
G. S. HAND, JR.	University of North Carolina
S. HAUSCHKA	The Johns Hopkins University
D. L. HESSEL	The Johns Hopkins University
B. HORWITZ	Emory University
M. R. LURIE	University of Miami
D. MOORE	University of Pennsylvania
M. C. REPORTER	Carnegie Institution of Washington
N. M. SCHULKIND	New York University, School of Medicine
A. E. S. SMITH	California Institute of Technology
E. L. STERN	University of Chicago
D. T. SULLIVAN	The Johns Hopkins University

IV. LECTURES

D. SZOLLOSI	Ultrastructural Studies on Fertilization and the Gametes
R. A. BEATTY	Genetic Effect on Gametes
R. MAGGIO	Activation of Protein Synthesis in the Sea Urchin Egg at Fertilization
H. MOHRI	Mitochondrial Functions of Bull Spermatozoa
P. M. BHARGAVE	Ribonucleic Acid and the Amino Acid Incorporation in Spermatozoa
Y. HIRAMOTO	Mechanical Properties of the Protoplasm of the Sea Urchin
R. RIKMENSPOEL	Biophysical Approaches to Problems of Spermatozoan Motility
R. YANAGIMACHI	The Hamster as a Material for the Study of Fertilization
R. C. RUSTAD	Radiation Effects in Sea Urchin Gametes
L. WEISS	Interactions Between Cells Making Contact

Neurophysiology Training Program

I. Instructors

S. W. Kuffler	Harvard Medical School, in charge of program
E. J. Furshpan	Harvard Medical School
J. G. Nicholls	Harvard Medical School

II. Assistants

R. Bosler	Harvard Medical School

(No lectures given—only seminars)

III. Students

J. M. Camhi	Harvard Medical School
A. M. Friedlander	University of Pittsburgh
J. Harvey	The Johns Hopkins University
J. S. McReynolds	Harvard Medical School
R. Pipkin	Harvard Medical School
P. Sterling	Western Reserve University
B. Wickelgren	Massachusetts Institute of Technology

Comparative Physiology Training Program

I. Instructors

L. Kleinholz	Reed College, in charge of program
B. C. Abbott	University of Illinois
A. Janoff	New York University
G. Kaley	New York University
B. Zweifach	New York University

II. Assistants

F. Kimball	Reed College
J. C. Munday	University of Illinois

III. Students

E. A. Ashby	University of Texas
G. M. Connell	Indiana University
G. A. Cottrell	Harvard University
P. J. Dowd	Vestibular Laboratory
C. R. Jones	Fordham University
W. R. Kem	University of Illinois
M. J. Pak	University of Minnesota
P. Stern	University of Michigan

IV. Lectures

L. Kleinholz	Neurosecretion and Endocrine Physiology
G. Kaley	Cardiovascular Physiology
A. Janoff	Comparative Aspects of Lysosome Function & Comparative Aspects of Leucocyte Physiology
B. C. Abbott	Excitation-Contraction Coupling and Relaxing Factor in Muscle
F. A. Brown, Jr.	A Unified Clock Theory
C. Read	Comparative Aspects of Membrane Transport
R. Allen	Cell Movement
G. Cottrell	Binding of Biologically-Active Substances

6. TABULAR VIEW OF ATTENDANCE, 1960–1964

	1960	*1961*	*1962*	*1963*	*1964*
INVESTIGATORS—TOTAL	458	458	494	490	512
Independent	273	256	279	261	273
Library Readers	50	49	56	51	47
Research Assistants	135	151	159	178	192
STUDENTS—TOTAL	122	130	121	124	126
Invertebrate Zoology	49	40	38	40	40
Embryology	20	21	20	20	20
Physiology	28	28	28	28	30
Botany	18	19	20	20	19
Ecology	13	22	15	16	17
TRAINEES—TOTAL					30
Nerve-Muscle					7
Comparative Physiology					7
Fertilization and Gamete					16
TOTAL ATTENDANCE	580	586	615	614	668
Less persons represented in two categories	2	1	4	5	7
	578	585	611	609	661
INSTITUTIONS REPRESENTED—TOTAL	144	132	118	120	140
By Investigators	83	107	81	83	117
By Students	61	70	57	73	23
SCHOOLS AND ACADEMIES REPRESENTED					
By Investigators	5	3	3	4	0
By Students	2	0	2	0	0
FOREIGN INSTITUTIONS REPRESENTED	14	28	31	21	32
By Investigators	11	21	17	15	28
By Students	3	7	14	6	4

7. INSTITUTIONS REPRESENTED, 1964

Abbott Laboratories
Agnes Scott College
Albert Einstein Medical School
Allegheny College
American Museum of Natural History
Amherst College
Argonne National Laboratory
Arizona, University of
Beloit College
Boston University
Bowdoin College
Bowling Green State University
Brandeis University
Brooklyn College
Brown University
California, University of, Los Angeles
California, University of, Berkeley
California, University of, Livermore
California, University of, Santa Barbara
Capitol Radio Engineering Institute
Carnegie Institution of Washington
Catholic University of America
Chicago, University of, Medical School
Chicago, University of
Cincinnati, University of
Cornell University
Cornell University, Medical College
Dartmouth College
Dartmouth Medical School
Delaware, University of
Drew University
Duke University
duPont de Nemours & Company
Earlham College
Emory University
Florida State University
Florida, University of
Fordham University
Goucher College
Hahnemann Medical School
Harvard University
Harvard University Medical School
Haskins Laboratories
Hawaii, University of
Illinois, University of
Indiana University
Institute for Muscle Research
Iowa State University

Jefferson Medical College
Johns Hopkins University, The
Johns Hopkins University School of Medicine, The
Lafayette College
Lerner Marine Laboratory, of the American Museum of Natural History
Maine Vocational Technical Institute
Marquette University
Maryland, University of
Massachusetts General Hospital
Massachusetts Institute of Technology
Massachusetts, University of
Mellon Institute
Miami, University of
Michigan, University of
Minnesota, University of
Missouri, University of, Medical School
Morgan State College
Mount Holyoke College
Mount Sinai Hospital, The
National Institutes of Health
New York State University, College of Medicine at Brooklyn
New York State University, College of Medicine at Syracuse
New York University, Bellevue Medical Center
New York University, School of Dentistry
New York University, Washington Square College
North Carolina State of the University of North Carolina at Raleigh
North Carolina, University of
Northwestern University
Notre Dame, University of
Oak Ridge National Laboratory
Oberlin College
Oglethorpe University
Oregon Regional Primate Research Center
Oregon State University
Oregon, University of
Pembroke College
Pennsylvania, University of
Pennsylvania Medical School, University of
Pittsburgh, University of
Princeton University
Purdue University
Queens College
Reed College
Rensselaer Polytechnic Institute
Rhode Island, University of
Rice University
Ripon College
Rochester, University of, School of Medicine and Dentistry
Rockefeller Institute, The
Russell Sage College
Rutgers, The State University
Saint Louis University
Seton Hill College
Single Cell Research Foundation, Inc.
Smith College
South Florida College
Stanford University
Stanford University, School of Medicine
State University of New York at Buffalo
Swarthmore College
Syracuse University
Temple University
Tennesee, University of
Tufts University
Tulane University
Vassar College
Vermont, University of
Veterans Administration Hospital
Vanderbilt University
Virginia, University of
Washington University, at St. Louis
Wellesley College
Wesleyan University
Western Reserve University
Western Reserve University, School of Medicine
Wheaton College
Wilson College
Wisconsin, University of
Yale University

FOREIGN INSTITUTIONS REPRESENTED, 1964

Agricultural Research Council of Great Britain
Anatomisches Institut, der Universität Zurich, Switzerland
Biologische Anstalt Helgoland, Germany
Brussels University, Brussels
Dalhousie University, Halifax
Erlangen, University of, Germany
Gunma University, Japan
Leicester University, England
Ljubljana University, Yugoslavia
Medical Research Council of Great Britain
McMaster University, Canada
Pahlavi, University of, Shiraz, Iran
Queen's University, Ontario, Canada
Tokyo, University of, Japan
University College, London, England
University of Palermo, Italy
Utrecht, University of, Holland

Supporting Institutions, Agencies, and Individuals

Abbott Laboratories
Associates of the Marine Biological Laboratory
Atomic Energy Commission
CIBA Corporation
The Commonwealth Fund
Josephine B. Crane Foundation
Dr. William D. Curtis
The Ford Foundation
Dr. and Mrs. David W. Gaiser
The Grass Foundation
Mr. and Mrs. William H. Greer, Jr.
Dr. Ethel Browne Harvey
Mr. and Mrs. George F. Jewett, Jr.
The Lalor Foundation
Mrs. Grace T. Mast
Olin Matheson Charitable Trust
National Institutes of Health
National Science Foundation
Office of Naval Research
The Rockefeller Foundation
Schering Foundation, Inc.
Scientific American, Inc.
Mr. Gerard Swope, Jr.
The Upjohn Company
Wallace Laboratories
Mr. James H. Wickersham

8. Friday Evening Lectures, 1964

July 3	Hans Leo Kornberg The University of Leicester	Anaplerotic Sequences in Microbial Metabolism: Their Significance and Regulation
July 9, Thursday	Ricardo Miledi University College, London Alexander Forbes Lecturer at the MBL	Localization of Acetylcholine-Receptors and Cholinesterase in Muscle Fibres, Part I
July 10	Ricardo Miledi	Localization of Acetylcholine-Receptors and Cholinesterase in Muscle Fibres, Part II
July 17	Irwin R. Konigsberg Carnegie Institution of Washington	Clonal Analysis of Myogenesis
July 24	Sidney W. Fox The Florida State University	Experimental Geosynthesis and a Theory of Cellular Origins
July 31	Melbourne R. Carriker MBL	Hard Tissue Destruction by Marine Predatory Gastropods
August 7	Sol Spiegelman University of Illinois	The Transcription and Translation of Genetic Messages
August 14	R. Alan Beatty University of Edinburgh Senior Lalor Fellow at the MBL	The Gamete as a Microorganism
August 21	Ernst Hadorn University of Zurich F. R. Lillie Fellow at the MBL	New Patterns of Differentiation Arising in Permanent Cultures of *Drosophila* Cells *in vivo*
August 28	Kenneth D. Roeder Tufts University	What a Moth's Ear Tells its Nervous System about Bats

9. Tuesday Evening Seminars, 1964

July 7	H. Schuel L. Lorand R. Schuel	Isolation of Muscle-Relaxing Particles with the Zonal Centrifuges
	J. S. Nagle	Differential Sorting of Shells in the Swash Zone
	C. C. Speidel R. H. Cheney	Deviations in Motility of Developing Sea Urchins Induced by Irradiation (film)
July 14	J. Cohen G. Szabó	The Transfer of Melanin Granules from Melanocytes into Malpighian cells of the Mammalian Epidermis (illustrated with timelapse photography)
	S. Karasaki	The Sites of Nuclear RNA Synthesis during Amphibian Embryogenesis
	R. Alan Beatty	Density Gradient Media for Spermatozoa
July 21	S. Higashino	Analysis of Biological Excitable Membrane by Means of Voltage-Current-Time Characteristics
	A. M. Zimmerman L. Silberman	Further Studies on Incorporation of H^3 Thymidine in *Arbacia* Eggs Under Hydrostatic Pressure
	D. Marsland	High Pressure Reversal of the Anti-Mitotic Effects of Heavy Water (D_2O)
July 28	A. Janoff B. Zweifach	Production of Inflammatory Changes in the Micro-Circulation by Cationic Proteins Extracted from Lysosomes
	H. Sato S. Inoué	Condensation of the Sperm Nucleus and Orientation of DNA Molecules during Spermiogenesis in *Loligo pealii*
	P. Person	Cartilage in a Marine Polychaete *Eudistylia*
August 4	W. E. Love	Microheterogeneity in the Hemoglobin from Individual Sea Lampreys
	Y. Hiramoto	Further Studies on the Cell Division without Mitotic Apparatus in Sea Urchin Eggs
	A. B. Novikoff	GERL, its Form and Function in Neurons of Rat Spinal Ganglia
August 11	D. Bownds G. Wald	The Reaction of Rhodopsin and its Derivative with Sodium Borohydride
	S. Zigman S. Lerman	A Cold-Precipitable Protein in the Dogfish Lens
August 18	W. Auclair	On the Chromosome Number of *Arbacia*
	V. Zullo	Re-evaluation of the Late Cenozoic Cirriped, "Tamiosoma" Conrad
	R. Parker A. Driscoll J. S. Nagle K. Lukas	Preliminary Quantitative Study of Small-Scale Environmental and Faunal Variability

10. Members of the Corporation, 1964

Life Members

Adolph, Dr. Edward F., University of Rochester, School of Medicine & Dentistry, Rochester, New York
Brodie, Mr. Donald, 522 Fifth Avenue, New York 18, New York
Cole, Dr. Elbert C., 2 Chipman Park, Middlebury, Vermont
Cowdry, Dr. E. V., 4580 Scott Avenue, St. Louis 10, Missouri
Crane, Mrs. W. Murray, 820 Fifth Avenue, New York 21, New York
Hess, Dr. Walter, 286 North Fairview Avenue, Spartanburg, South Carolina
Hisaw, Dr. F. L., Biological Laboratories, Harvard University, Cambridge 38, Massachusetts
Irving, Laurence, University of Alaska, College, Alaska
Jacobs, Dr. M. H., Department of Physiology, University of Pennsylvania, Philadelphia 4, Pennsylvania
Lowther, Dr. Florence, Barnard College, New York 21, New York
MacDougall, Dr. Mary Stuart, Mt. Vernon Apartments, 423 Clairmont Avenue, Decatur, Georgia
Malone, Dr. E. F., 6610 North 11th Street, Philadelphia 26, Pennsylvania
Means, Dr. J. H., 15 Chestnut Street, Boston Massachusetts
Medes, Dr. Grace, 303 Abington Avenue, Philadelphia 11, Pennsylvania
Moore, Dr. J. Percy, RD No. 1, Box 437, Chapel Hill, North Carolina
Payne, Dr. Fernandus, Indiana University, Bloomington, Indiana
Plough, Dr. H. H., Amherst College, Amherst, Massachusetts
Porter, Dr. H. C., University of Pennsylvania, Philadelphia 4, Pennsylvania
Scott, Dr. Ernest L., Columbia University, New York 21, New York
Schrader, Dr. Sally, Duke University, Durham, North Carolina
Turner, Dr. C. L., Northwestern University, Evanston, Illinois
Waite, Dr. F. G., 144 Locust Street, Dover, New Hampshire
Wallace, Dr. Louise B., 359 Lytton Avenue, Palo Alto, California
Warren, Dr. Herbert S., 2768 Egypt Road, Audubon, Pennsylvania
Whedon, Dr. A. D., 21 Lawncrest, Danbury, Connecticut

Regular Members

Abell, Dr. Richard G., 55 East 2nd Avenue, New York 28, New York
Adelberg, Dr. Edward A., Department of Microbiology, Yale University, New Haven, Connecticut 06520
Adelman, Dr. William J., Department of Physiology, University of Maryland Medical School, Baltimore 1, Maryland
Albert, Dr. Alexander, Mayo Clinic, Rochester, Minnesota
Allen, Dr. M. Jean, Department of Biology, Wilson College, Chambersburg, Pennsylvania
Allen, Dr. Robert D., Department of Biology, Princeton University, Princeton, New Jersey 08540
Alscher, Dr. Ruth, Department of Physiology, Manhattanville College, Purchase, New York

Amatniek, Dr. Ernest, 34 Horner Avenue, Hastings-on-the-Hudson, New York
Amberson, Dr. William R., Katy Hatchs Road, Falmouth, Massachusetts
Anderson, Dr. J. M., Department of Zoology, Cornell University, Ithaca, New York
Anderson, Dr. Rubert S., Medical Laboratories, Army Chemical Center, Maryland
Armstrong, Dr. Philip B., Department of Anatomy, State University of New York, College of Medicine, Syracuse 10, New York
Arnold, Dr. John Miller, Department of Zoology, Iowa State University, Ames, Iowa 50010
Arnold, Dr. William A., Division of Biology, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831
Atwood, Dr. Kimball C., 702 West Pennsylvania Avenue, Urbana, Illinois
Auclair, Dr. Walter, Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio 45221
Austin, Dr. Colin Russell, Delta Regional Primate Research Center, Covington, Louisiana 70433
Austin, Dr. Mary L., 506½ North Indiana Avenue, Bloomington, Indiana
Ayers, Dr. John C., Department of Zoology, University of Michigan, Ann Arbor, Michigan
Baitsell, Dr. George A., Osborn Memorial Laboratories, Yale University, New Haven, Connecticut 06520
Ball, Dr. Eric G., Department of Biological Chemistry, Harvard Medical School, Boston 15, Massachusetts
Ballard, Dr. William W., Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire
Baltus, Dr. Elyane, Laboratoire de Morphologie Animale, 1850 Chaussée de Wavre, Bruxelles 16, Belgique
Bang, Dr. F. B., Department of Pathobiology, The Johns Hopkins University School of Hygiene, Baltimore 5, Maryland
Bard, Dr. Phillip, The Johns Hopkins Medical School, Baltimore 5, Maryland
Barth, Dr. L. G., Marine Biological Laboratory, Woods Hole, Massachusetts 02543
Barth, Dr. Lucena, Marine Biological Laboratory, Woods Hole, Massachusetts 02543
Bartlett, Dr. James H., Department of Physics, University of Illinois, Urbana, Illinois
Bauer, Dr. Eric G., Department of Anatomy, University of Minnesota, Minneapolis, Minnesota
Baylor, Dr. E. R., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543
Baylor, Dr. Martha B., Marine Biological Laboratory, Woods Hole, Massachusetts 02543
Beams, Dr. Harold W., Department of Zoology, State University of Iowa, Iowa City, Iowa
Beck, Dr. L. V., Department of Pharmacology, Indiana University, School of Experimental Medicine, Bloomington, Indiana

Behre, Dr. Elinor M., Black Mountain, North Carolina
Bell, Dr. Eugene, Department of Biology, Massachusetts Institute of Technology, Cambridge 39, Massachusetts
Bennett, Dr. Michael V. L., Department of Neurology, College of Physicians & Surgeons, New York 32, New York
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Bartow, Mrs. Philip
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V. REPORT OF THE LIBRARIAN

The summer of 1964 saw many physical changes in the Library. The older section of the stacks was repainted, new tile flooring put down on all five floors, and lighting changed completely to a fluorescent system. New lights were placed on all tables in the wing and the reserve desks now contain file cabinets. A large catalog room, three new offices for the staff, and a Xerox room were added to the existing space. Also, for the first time, all of our most valuable books were brought together in a Rare Books Room, which is lined with floor-to-ceiling paneled shelving and contains as of this date 517 volumes. This room is located within the offices and is available only while the staff is on duty.

During the year we received and serviced 844 requests on Interlibrary Loan, and we requested 137 titles for our use here. In the Fall, 1545 volumes were sent to the bindery. A very generous gift of nearly 2000 reprints was received from Dr. William Randolph Taylor. The total number of bound volumes in the Library is now approximately 100,000.

The Library holdings for this year are:

Total Number of Serial Titles in Library	3567
Number Received Currently	1917
On Subscription	788
On Exchange (with Biol. Bull., Coll. Reprints)	874
On Gift Basis	255
Total Number of Books in Library	15,384
Number Added in 1964	462
Received From Book Exhibit	101
Total Number of Reprints	231,351
Number Added in 1964	3351

Jane Fessenden,
Librarian

VI. REPORT OF THE TREASURER

The market value of the General Endowment Fund and the Library Fund at December 31, 1964, amounted to $2,370,890 as against book value of $1,235,860. This compares with values of $2,155,489 and $1,242,896, respectively, at the end of the preceding year. The average yield on the Securities was 3.51% of the market value and 6.74% of the book value. The total uninvested principal cash in the above accounts as of December 31, 1964, was $5,595. Classification of the securities held in the Endowment Funds appears in the Auditor's report.

The market value of the pooled securities as of December 31, 1964 was $580,677 with uninvested principal cash of $2,210, the market value at December 31, 1963, being $390,026. The book value of the securities in this account was $562,547 on December 31, 1964, compared with $315,196 a year earlier. The average yield on market value was 3.6% and 4.6% of book value.

The proportionate interest in the Pool Fund Account of the various Funds as of December 31, 1964, is as follows:

Pension Funds	23.098%
General Laboratory Investment	32.264%
Other:	
Bio Club Scholarship Fund	.923%
Rev. Arsenius Boyer Scholarship Fund	1.130%
Gary N. Calkins Fund	1.059%
Allen R. Memhard Fund	.206%
F. R. Lillie Memorial Fund	3.566%
Lucretia Crocker Fund	3.860%
E. G. Conklin Fund	.652%
Jewett Memorial Fund	.343%
M. H. Jacobs Scholarship Fund	.465%
Anonymous Gift	1.221%
Herbert W. Rand Fellowship	23.326%
Mellon Foundation	7.887%

Donations from the MBL Associates for 1964 were $4,830.00 as compared with $4,295.00 for 1963. Unrestricted gifts from foundations, societies and companies amounted to $14,575.

During the year, we administered the following grants:

Investigators	*Training*	*MBL Institutional*
13 NIH	3 NIH	4 NIH
3 NSF	2 NSF	3 NSF
1 Ford		3 ONR
1 ONR		1 AEC
1 Commonwealth		1 Ford
19	5	12

The rate of overhead on grants to investigators is 20%, based on the amount expended. The overhead on these grants for this year amounted to $81,239 as compared with $56,494 for the preceding year.

The Lillie Fellowship Fund, with a market value of $114,293 and a book value of $92,893, as well as the investment in General Biological Supply House with book value of $12,700, is carried in the Balance Sheet item "Other Investments."

The General Biological Supply House fiscal year ended June 30, 1964, and had a profit after taxes of $309,651 as compared to $241,616 in 1963 and $302,657 in 1962 and $302,851 in 1961 and $314,034 in 1960.

In the fiscal year 1964 the Marine Biological Laboratory received dividends from the General Biological Supply House of $63,500 as against $42,164 in 1963, $38,100 in 1962, $33,020 in 1961, and $30,480 in 1960.

Following is a statement of the auditors:

To the Trustees of Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheet of Marine Biological Laboratory as at December 31, 1964, the related statement of operating expenditures, income and current fund and statement of funds for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We examined and have reported on financial statements of the Laboratory for the year ended December 31, 1964.

In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of Marine Biological Laboratory at December 31, 1964 and 1963 and the results of its operation for the years then ended on a consistent basis.

The supplementary schedules included in this report were obtained from the Laboratory's records in the course of our examination and in our opinion, are fairly stated in all material respects in relation to the financial statements, taken as a whole.

Boston, Massachusetts
March 26, 1965

LYBRAND, ROSS BROS. & MONTGOMERY
JAMES H. WICKERSHAM,
Treasurer

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1964 and 1963

Investments	*1964*	*1963*
Investments held by Trustee:		
Securities, at cost (approximate market quotation 1964—$2,370,890)	$1,235,860	$1,242,896
Cash	5,595	431
	1,241,455	1,243,327
Investments of other endowment and unrestricted funds:		
Pooled investments, at cost (approximate market quotation 1964—$579,161) less $5,728 temporary investment of current fund cash	479,344	309,468
Other investments	118,888	120,424
Cash	39,522	14,083
Accounts receivable	10,664	21,131
	$1,889,873	$1,708,433

Plant Assets		
Land, buildings, Library and equipment (note)	5,136,289	4,931,472
Less allowance for depreciation (note)	1,378,887	1,313,162
	3,757,402	3,618,310
Construction in progress	109,215	
Short-term investments, at cost	192,360	
	$4,058,977	$3,618,310

Current Assets		
Cash	79,637	100,991
Temporary investment in pooled securities	5,728	5,728
U. S. Treasury bills, at cost	96,045	74,324
Accounts receivable (U. S. Government, 1964—$84,793; 1963—$95,142)	138,489	140,825
Inventories of specimens and Bulletins	33,401	45,288
Prepaid insurance and other	6,844	7,062
	$ 360,144	$ 374,218

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1964 and 1963

Endowment Funds	*1964*	*1963*
Endowment funds given in trust for benefit of the Marine Biological Laboratory	$1,241,455	$1,243,327
Endowment funds for awards and scholarships:		
Principal	295,710	126,980
Unexpended income	14,289	12,077
	309,999	139,057
Unrestricted funds functioning as endowment	206,378	206,378
Retirement fund	123,298	108,481
Pooled investments—accumulated gain	8,743	11,190
	$1,889,873	$1,708,433

Plant Funds		
Funds expended for plant, less retirements	5,245,504	4,931,472
Less allowance for depreciation charged thereto	1,378,887	1,313,162
	3,866,617	3,618,310
Unexpended plant funds	192,360	
	$4,058,977	$3,618,310

Current Liabilities and Funds		
Accounts payable and accrued expenses	33,315	62,763
Advance subscriptions	10,926	10,362
Unexpended grants—research	99,000	110,433
Unexpended balances of gifts for designated purposes	17,995	13,531
Current fund	198,908	177,129
	$ 360,144	$ 374,218

Note.—The Laboratory has since January 1, 1916, provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF OPERATING EXPENDITURES, INCOME AND CURRENT FUND

Years Ended December 31, 1964 and 1963

Operating Expenditures

	1964	*1963*
Research and accessory services	$ 293,396	$ 273,333
Instruction	160,820	147,163
Library and publications (including book purchases—1964, $24,304; 1963, $25,628)	80,416	77,922
Direct costs on research grants	493,890	484,640
Direct costs on institution support grants	150,450	129,642
	1,178,972	1,112,700
Administration and general	112,453	97,339
Plant operation and maintenance	139,684	111,441
Dormitories and dining	175,696	174,030
Additions to plant from current income	5,045	7,793
	1,611,850	1,503,303
Less depreciation included in plant operation and dormitories and dining above but charged to plant funds	67,437	67,472
	1,544,413	1,435,831

Income

	1964	*1963*
Research fees	88,240	113,024
Accessory services (including sales of biological specimens—1964, $42,051; 1963, $38,019)	114,181	103,398
Instruction fees	28,470	28,461
Library fees, Bulletins, subscriptions and other	49,393	43,952
Dormitories and dining income	133,759	129,486
Grants for support of institutional activities:		
Instruction and training	138,540	131,564
Support services	150,450	129,642
General	109,219	113,853
Reimbursements and allowances for direct and indirect costs on specific research grants	575,129	541,134
Gifts used for current expenses	22,135	29,285
Investment income used for current expenses	156,676	134,591
	1,566,192	1,498,390
Excess current income	21,779	62,559
Current fund balance January 1	177,129	114,570
Current fund balance December 31	$ 198,908	$ 177,129

MARINE BIOLOGICAL LABORATORY

STATEMENT OF FUNDS

Year Ended December 31, 1964

	Balance January 1, 1964	*Gifts and Other Receipts*	*Investment Income*	*Used for Current Expenses*	*Other Expenditures*	*Balance December 31, 1964*
Invested funds	$1,708,433	$ 187,132	$164,448	$ 153,890	$ 16,250	$1,889,873
Unexpended plant funds		452,800	1,575		262,015	$ 192,360
Unexpended research grants	$ 110,433	1,110,905		1,122,338		$ 99,000
Unexpended gifts for designated purposes	$ 13,531	29,687		22,135	3,088	$ 17,995
Current fund	$ 177,129	21,779(1)				$ 198,908
		$1,802,303	$166,023	$1,298,363	$281,353	
Gifts		198,447				
Grant for facilities		452,800				
Grants for research, training and support		1,110,905				
Appropriated from current income and other		22,722				
Net loss on sale of securities		(4,350)				
Excess of current income over expenditures		21,779(1)				
		$1,802,303				
Expended for construction and renovation of facilities					262,015	
Scholarship awards					4,124	
Payments to pensioners					12,126	
Other					3,088	
					$281,353	

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1964

Securities held by Trustee:	*Cost*	*% of Total*	*Market Quotations*	*% of Total*	*Investment Income 1964*
General endowment fund:					
U. S. Government securities	$ 92,310	8.9	$ 91,086	4.6	$ 4,449
Corporate bonds..........	528,638	51.1	522,095	26.5	21,177
Preferred stocks..........	54,422	5.3	53,200	2.7	2,186
Common stocks...........	358,452	34.7	1,306,140	66.2	42,950
	1,033,822	100.0	1,972,521	100.0	70,762
General Educational Board endowment fund:					
U. S. Government securities	18,086	8.9	17,836	4.5	1,221
Other bonds.............	107,310	53.2	109,091	27.4	4,106
Preferred stocks..........	15,641	7.7	15,100	3.8	700
Common stocks...........	61,001	30.2	256,342	64.3	6,508
	202,038	100.0	398,369	100.0	12,535
Total securities held by Trustee............	$1,235,860		$2,370,890		83,297
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government securities	61,005	12.6	59,730	10.3	2,308
Corporate bonds..........	125,736	25.9	125,370	21.7	4,782
Common stocks...........	298,331	61.5	394,061	68.0	7,489
	485,072	100.0	$ 579,161	100.0	14,579
Less temporary investment of current fund cash.....	(5,728)				(251)
	479,344				14,328
Other investments:					
U. S. Government securities	27,947				1,312
Other bonds.............	15,031				749
Preferred stocks..........	3,728				130
Common stocks...........	58,887				65,576
Real estate...............	13,295				
	118,888				67,767
Total investments of other endowment and unrestricted funds...	$ 598,232				82,095
Total investment income...........					165,392
Custodian's fees charged thereto....					(944)
Investment income distributed to funds.........................					164,448
Plant investments:					
U. S. Treasury bills, due 3/4/65.	80,085				684
Bank of New York acceptances, due January, 1965..........	112,275				891
	$ 192,360				1,575
Current investments:					
U. S. Treasury bills, due March 4 and October 31, 1965........	$ 96,045				2,686
Temporary investment in pooled securities..................	$ 5,728				251
					2,937
					$168,960

THE INDUCTIVE ROLE OF THE YOLK EPITHELIUM IN THE DEVELOPMENT OF THE SQUID, LOLIGO PEALII (LESUEUR)[1, 2]

JOHN M. ARNOLD

Marine Biological Laboratory, Woods Hole, Massachusetts 02543, and the Department of Zoology, University of Minnesota, Minneapolis, Minnesota 55455[3]

While a fairly large body of information exists on the descriptive aspects of the embryology of the cephalopods, relatively little experimental work has been done on these animals. These embryos seem superficially to be ideally suited to embryological analysis since they are large, abundant, and are relatively easy to obtain (Arnold, 1962). One of the major difficulties has been the inability to work with embryos outside of the chorion. Ranzi (1931) tried to raise *Sepia officinalis* in sea water but had little success. He was able to isolate various organs and parts of the embryos of *Sepia* by operating through the chorion, and concluded that isolated organs and tissues were capable of self-differentiation. This technique severely limited operative procedures, and he was forced to use rather late stages, (Naef stage XII = Arnold stage 26) in which most of the organ primordia were rather well formed.

In the summer of 1961 it was possible to devise culture techniques by which the dechorionated embryos would survive and develop normally. This has allowed some experimental analysis of young embryos of the common Atlantic coast squid, *Loligo pealii.*

MATERIALS AND METHODS

Since the techniques used in this study have not been fully described, it is necessary to give a detailed account of the procedures used. Most of the experiments were performed *in vitro* in a culture medium made of three basic components: whole adult squid blood, sterile sea water, and an antibiotic stock solution. The blood was drawn under sterile conditions from the vena cava of the adults and stored frozen in 3-ml. glass tubes. Before use it was thawed and diluted with an equal volume of sterile sea water. To this was added about 1% of an antibiotic stock solution composed of 0.5% streptomycin, 0.05% phenol red, and 50,000 units of potassium penicillin G in double-distilled water which had been saturated with sulfadiazine. The embryos were cultured in glass vessels made expressly for this purpose. Each of these vessels had a volume of about 0.1 ml. and had two small depressions in the bottom for holding the embryos. This culture vessel was placed

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[1] This work is a portion of a thesis submitted in partial fulfillment of the Ph.D. requirements of the Department of Zoology, University of Minnesota.

[2] Part of this work was done while the author was the recipient of an N.S.F. Predoctoral Fellowship.

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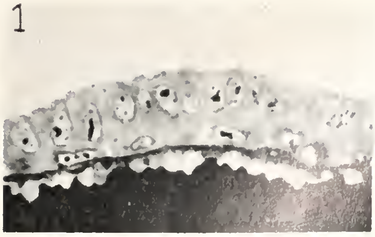
in a covered preparation dish to which a few drops of sterile sea water had been added to saturate the atmosphere and maintain osmotic equilibrium with the medium in the vessel.

The embryos were prepared for culture by stripping off the outer tunics of the egg string with the fingers, cutting up the denuded string and transferring individual embryos in their chorions through two changes of sterile sea water. The chorion was then torn open and the embryo washed twice in sterile sea water. The operative procedures described below were performed and the embryo was then transferred to the freshly prepared medium. The embryos were incubated at 18° C. (± 0.5° C.) and examined twice daily. The medium was changed every second day when the experiments exceeded this length of time.

Under these conditions the embryos appeared to develop completely normally when compared with control embryos still in their chorions and in normal sea water. The cultured embryos seemed to develop slightly faster than the controls, possibly because of greater availability of oxygen due to the lack of the chorion. Some embryos were maintained for 178 hours in culture with no apparent adverse effects. If the medium was not changed at least every three days, development would slow down. This effect was reversed by the addition of fresh medium.

Parts of the stage 16 and 17 embryos (Arnold, 1965) were removed with stainless steel needles before culturing. At these stages, the cephalopod embryo consists essentially of an outer layer of cells (future body of the embryo), a layer of greatly flattened cells (yolk epithelium), and a central mass of yolk. The various organ primordia could be identified by the thickened nature of the outer layer of cells (columnar *vs.* cuboidal cells) and the asymmetrical nature of the egg. Most of the operations involved removal of the parts of the eye, but the same confirming experiments were also done on the otocyst, arms and funnel folds. Three different types of operations were performed. The first involved the removal of the whole eye primordium, including some of the underlying yolk. This isolate would round up and the yolk would become incorporated within the center. These isolates survived quite well in the culture medium and underwent considerable differentiation. The second operation separated the outer layer of cells from the yolk epithelium. This was accomplished by gently rubbing the cells with the blunt edge of a needle until the cells became loosened and somewhat sticky. The outer layer of cells could then be caught on the point of a needle and pulled off in a sheet. A second needle was used to cut the sheet of cells well beyond the organ primordium. With practice, about one-fourth to one-third of the total surface of the outer cells could be removed and the embryo transferred to culture medium with only a few minutes exposure to sterile sea water. Sections of unstripped and stripped embryos are shown in Figures 1 and 4. In a few cases the eye anlage was removed by the technique described below for dissociating cells. A large number of embryos was examined and appropriate ones were selected. In the third operation the outer layer of cells was stripped and a small portion of the yolk epithelium removed. This was rather hard to accomplish since the embryos tended to lose yolk from the wound, which mechanically inhibited wound healing. However, enough cases were successful so interpretable results were obtained. In a few cases masses of cells were grafted onto the freshly stripped yolk epithelium. These cells were obtained by cutting up an egg string and shaking it in sea water adjusted to pH 5 with 1 N HCl

1



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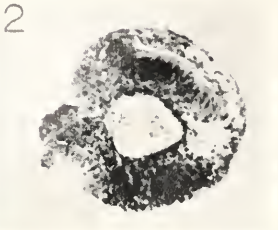

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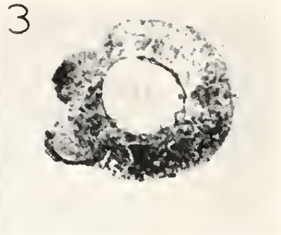

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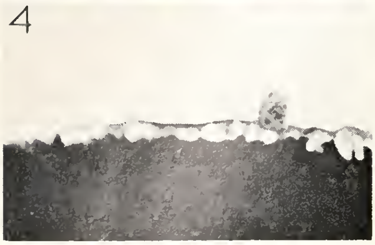

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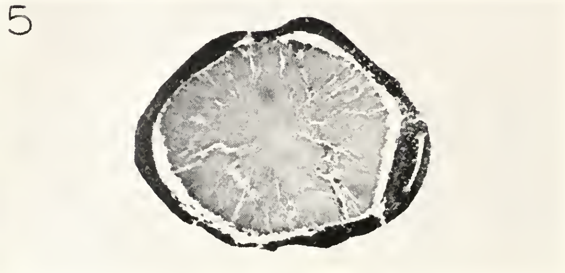

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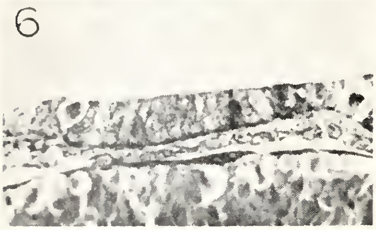

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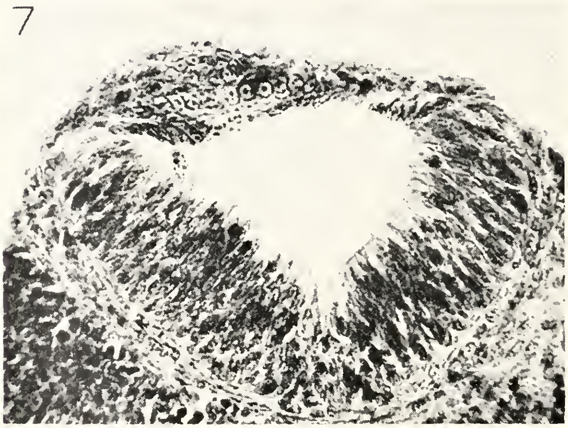

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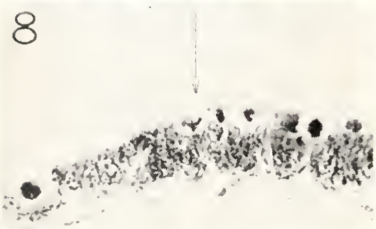

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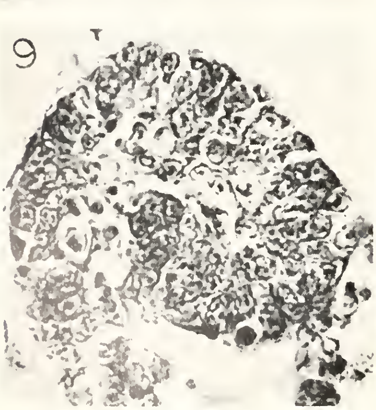

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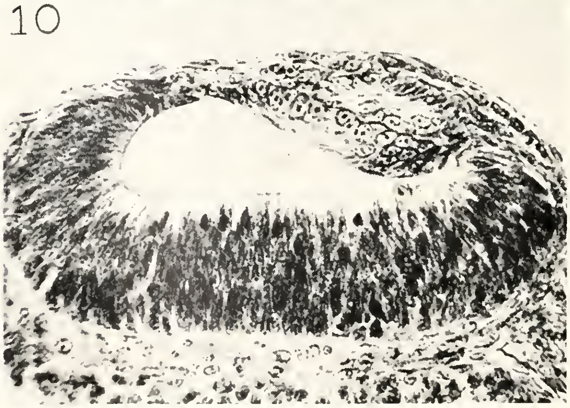

Figures 1–10.

until the egg-jelly dissolved. Once the egg-jelly was completely dissolved and the embryos inside their chorion were completely free, the embryos were shaken violently for about seven to ten minutes. The outer layer of cells was dissociated from the embryos, and usually single cells or small groups of cells resulted. The embryos were immediately examined, and those which appeared to be well dissociated were selected. At stage 17 the dissociated cells would reaggregate and form small solid clumps in about 15 minutes. These small clumps would fuse until about seven to fifteen aggregates remained in each chorion. Although these clumps would remain alive for extended periods, no organ or tissue differentiation was ever observed. Newly formed aggregates (one to two hours after dissociation) were stained in a solution of neutral red (0.01%) and grafted onto the freshly stripped yolk epithelium. The aggregates stuck quite readily and tended to flatten out on the surface of the embryo. The aggregates remained visible and were easily distinguished from the cells of the host embryo by their dye content.

RESULTS

When the whole eye primordium was removed, together with some of the surrounding tissue and underlying yolk, the resultant isolate rounded up with the yolk on the inside. These isolates differentiated quite normally and complete organs were formed (Figs. 2 and 3). The wound on the donor embryo lost a portion of yolk, but eventually the edges of the wound closed, development continued, and the unoperated organs differentiated normally. There was no evidence of any regeneration or replacement of the organs removed (Fig. 5). A total of 25 operations was performed on stage 17, and 13 operations on stage 16 embryos. In all cases the results of these operations were the same. The possibility of inhibition of development due to operative trauma was checked by a series of sham operations in which either less than the whole eye primordium was removed or a few cuts were made in the surface of the embryo. In these cases there was loss of yolk at the site of the wound but after healing normal development ensued.

Removal of the outer layer of cells resulted in a different pattern of development. The isolated cells usually formed a small clump that showed no further differentiation and lost the characteristics formerly possessed. These small clumps of cells did not survive well, possibly due to the trauma of surgery. Of those that

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FIGURE 1. Section of the eye region of a stage 17 embryo. Note the yolk epithelium and the columnar nature of the outer layer of cells. Epon 812, Azure II-methylene blue; 660 ×.

FIGURE 2. Isolated organs of the embryo shown in Figure 5. Both the outer layer of cells and the yolk epithelium were isolated; *ca.* 190 ×.

FIGURE 3. Lower section of the same isolate as in Figure 2. In culture 45 hours.

FIGURE 4. Section of the yolk epithelium after removal of the outer layer of cells. One yolk epithelium cell has rounded up abnormally above the rest of the yolk epithelium; 660 ×.

FIGURE 5. Donor embryo for Figures 2 and 3. Note lack of organs on one side.

FIGURE 6. Regenerated otocyst after removal of the outer layer of cells; 900 ×.

FIGURE 7. Control eye for Figure 10; 725 ×.

FIGURE 8. Section through the retina of a stripped embryo onto which dissociated-reaggregated cells were grafted. In culture 40 hours; 1000 ×.

FIGURE 9. Dissociated-reaggregated cells after 55 hours. Note the lack of any tissue differentiation; 1000 ×.

FIGURE 10. Regenerated eye after removal of only the outer layer of cells. Compare with Figure 7.

did survive, no evidence of differentiation could be detected. The wound produced by this operation was closed by migration of the surrounding outer layer of cells over the denuded yolk epithelium. In covering the yolk epithelium the cells would cut off any small blebs of yolk that occasionally resulted from small punctures accidentally produced in the yolk epithelium during the operation. That these cells of the outer layer actually migrated over the wound rather than grew there was demonstrated by the speed at which the covering took place (one to two hours) as well as by a series of careful observations during wound closure. A total of 21 stage 17 and 10 stage 16 embryos was operated on in this fashion and all were cultured for at least 45 hours. In all of these cases the cells which migrated over the stripped yolk epithelium differentiated with the structures that would have normally differentiated in that site. In some cases (8) one-third of the total outer layer of cells was removed, yet the resultant embryos had all of their organs when examined and sectioned after approximately 48 hours in culture (Figs. 7 and 10). There seemed to be a slight retardation of development of the replaced organs but otherwise the course of development corresponded exactly to that of the unoperated side of the embryo. The eye, otocyst, arms and funnel folds could be thus stripped off and replaced by the cells normally destined to form other organs (*e.g.*, Fig. 6).

Since the above results would implicate the yolk epithelium as possibly having a causal role in the differentiation of the overlying cells, two other experiments were attempted to test this hypothesis. The first of these involved stripping off the outer layer of cells of stage 17 embryos and removing a small portion of the yolk epithelium. This was not easily accomplished because the yolk epithelium was easily torn. However, in two cases the yolk epithelium in the future region of the otocyst was successfully removed and development was carefully followed. In this case closure of the wound proceeded as before and differentiation of the organs followed but the embryo lacked the otocyst on the operated side. The second test involved grafting dissociated-reaggregated cells onto the freshly denuded yolk epithelium. Normally these aggregated cells survived in the culture medium for extended periods and gradually died and disintegrated. In no case did any of the aggregates resulting from well dissociated embryos ever show any interpretable differentiation (Fig. 9). When these cells were grafted onto the denuded yolk epithelium, they stuck quite readily and spread out slightly. The position and extent of the grafted cells could be easily ascertained by the dye they contained. When sectioned it appeared that those cells in contact with the yolk epithelium differentiated into rather normal-looking tissue which corresponded to the location on the embryo (Fig. 8). In the case of the primordial retina, the cells became columnar in appearance, underwent mitosis in the characteristic position, and appeared to be well incorporated in the host organ. In eight successful cases, parts of four grafts unquestionably became incorporated into the developing retina. In the remaining case the results were not as clearcut because of yolk leakage at the site of the graft.

The possibility of the yolk playing a significant specific causal role in the differentiation was eliminated by experimentally removing up to one-half of the yolk of the embryo. Despite this rather large loss in volume the embryos differentiated normally but were reduced in size, particularly in the yolk sac region. These results agree with those obtained by Okada (1927) on *Loligo bleekeri*.

DISCUSSION

The results reported have led the author to the conclusion that the cells of the outer layer are indeterminate in their fate unless influenced by the underlying yolk epithelium. This appears to fit the classic ideas of embryonic induction, with the yolk epithelium being the inductor and the outer layer of cells responding to its inductive influence. This can be stated with certainty for the eye and otocyst and as probable for some of the other organs (funnel folds, arms and gills). Organs other than these remain to be tested, but preliminary experiments indicate that their development is of the same general nature. It appears, therefore, that all of the organs of the embryonic body are laid out in inductive areas of the yolk epithelium and are fairly localized. This can be visualized as a "morphogenetic inductive map" in which developmental information is transferred to the highly labile or indifferent cells that overlie it. One of the rather unique features of this inductive map is its two-dimensional nature. Unlike many other systems in which induction occurs within a mass of heterogeneous tissue, the inductor in this case is present as a sheet. This system, therefore, should be more amenable to experimental analysis because the presumptive areas of the embryo can be rather easily localized and subjected to direct experimental analysis. Preliminary experiments with chemical treatment of the denuded yolk epithelium offer encouragement along these lines.

The problem of how to fit these results with the classical ideas of mosaic development of the molluscs is an open question. It is obvious that part of the embryo (the yolk epithelium) is rather rigidly fixed in its fate while the outer layer of cells is quite labile and subject to the inductive influences of the yolk epithelium. Just when and how this developmental information arises is still unknown but preliminary experiments indicate that the egg cortex of this embryo is also rather rigidly patterned and the morphogenetic patterns would then be referred ultimately to the ovary. Obviously, further work along these lines is indicated.

I would like to thank Drs. K. C. Shaw and A. L. Allenspach for reading this manuscript. Special thanks are due to Dr. Nelson T. Spratt, Jr. for his help and guidance with this work.

SUMMARY

1. Techniques for the *in vitro* culture of dechorionated *Loligo pealii* embryos, involving the use of whole adult squid blood, sterile sea water, and antibiotics, have been devised. Essentially normal development will take place in this culture medium.

2. The embryos in stages 16 and 17 are composed essentially of three components: an outer layer of cells, an inner cellular yolk epithelium, and the central mass of yolk. When the outer layer of cells and the yolk epithelium are isolated together, normal histogenesis and development will occur. When the outer layer of cells is isolated by itself, no differentiation occurs. This leads to the conclusion that the yolk epithelium induces the outer layer of cells to differentiate. This conclusion was upheld by grafting and deletion experiments.

3. The role of the yolk epithelium, therefore, may be in acting as a "morphogenetic inductive map."

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PHASE-SHIFTING A LUNAR RHYTHM IN PLANARIANS BY ALTERING THE HORIZONTAL MAGNETIC VECTOR [1]

FRANK A. BROWN, JR. AND YOUNG H. PARK

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It has been demonstrated that planarian worms directed initially northward in the late morning hours in an unvarying field-pattern of illumination will display a synodic monthly variation in their tendency to alter their course (Brown, 1962, 1963). From about September to March, the worms veer maximally to the left at new moon and to the right at full moon. During March and April the monthly rhythm typically becomes gradually transformed into a semi-monthly one with maxima in right-veering at both new and full moon, and left-veering at the moon's quarters.

The natural synodic month owes its existence, of course, to the periodic interference between the solar-daily and lunar-daily cycles, and hence it is reasonable to presume that the worms in the generation of their synodic monthly periodism, which remains phase-synchronized in a characteristic temporal relationship to the geophysical cycles, are depending upon responses to pervasive geophysical variations possessing both of these two important natural physical frequencies.

This monthly phenomenon was investigated under each of three experimental conditions on mornings during November through January, 1960–61. The conditions were: (1) controls in the unmodified ambient field, (2) augmenting the natural horizontal magnetic vector to 4.0 gauss, and (3) imposing a 4.0-gauss horizontal field in an east-west orientation. The monthly rhythm was conspicuously present in the natural field, absent in the augmented north-oriented magnetic field, and present but reduced in amplitude in the east-directed field. During these studies the observations of path direction on any given morning for each of the above conditions required an average of about 20 minutes to permit about 50 worm-paths to be recorded as an assay of the influence on that particular morning of each individual experimental condition.

The more recent demonstrations of after-effects of exposures of planarians to experimentally altered horizontal magnetic vectors with descriptions of their properties, together with suggestive variations in response dependent upon time of exposure to the experimental fields (Brown, 1965; Brown and Park, 1965), raised questions concerning what might be relationships of these effects and after-effects to influences of experimentally altered weak magnetic fields on the monthly rhythm of the worms.

The following report describes the results of an attempt to learn more concerning influences of experimental changes in strength and direction of the horizontal

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[1] This study was aided by grants from the National Science Foundation (G-15008) and the National Institutes of Health (GM-07405), and by a contract with the Office of Naval Research (1228-30).

magnetic vector upon the monthly variations in orientational tendencies and upon their generating mechanism.

MATERIALS AND METHODS

The data which were used in this investigation were the same as those employed for determining the duration of the after-effects of reversed horizontal magnetic vectors (Brown and Park, 1965). The data were obtained over the seventh-month period from October 8, 1963, through April 30, 1964. The detailed methods by which these data were derived and the primary reductions to which they were subjected have been described in the earlier report.

In essence, uniformly distributed over the 206-day period of the study, 360 series of observations were made, in each of which series an observer determined the average rate of turning of north-oriented worms for each of the ten 5-minute intervals of a continuous 50-minute period. For the first 10 minutes the worms were in the natural ambient geophysical field. Then during the next three consecutive 5-minute intervals, the worms remained continuously exposed to an experimentally reversed horizontal magnetic vector of 0.05 gauss. The final five 5-minute intervals followed return of the worms to the natural ambient field. Similarly, uniformly distributed over the same 206-day period, 357 50-minute series of observations were being made, just like the preceding series except that the reversed horizontal magnetic vector-strength was 4.0 gauss. Since an average of about 14 worm-paths could be recorded during a 5-minute period, about 50,000 individual worm-paths were recorded during the study. The mean path for each 5-minute interval while in the reversed magnetic field and following its removal was expressed as difference from the mean path for the initial 10-minute "controls," to obtain a measure of the response to the reversed fields. The response data were then reduced to mean responses to each field strength for each 5-minute interval for each of 41 consecutive 5-day periods of the study. Five-day means were used in order to reduce the size of the influence of day-to-day variations in uncontrolled geophysical factors.

For purposes of the present analysis, these data permitted one to determine the form of the mean monthly variation in the path of the initial "controls," and the form of any mean monthly variations which might be present in the turning responses of the worms to each of the two reversed field strengths. It was, finally, possible to follow and characterize any after-effects on such rhythmic responses which might tend to persist following the removal of the experimental fields.

Earlier studies had indicated that monthly variations occur in response to very weak magnetic fields. To disclose any monthly variation in response to the experimental fields of this study and in the after-effects, the data were used in the following manner. The mean response for each 5-day interval was treated as if it were a value obtained on that particular day over which the interval was centered. Thus, each 5-day group could be ascribed a specific day relative to new moon in the natural lunar monthly cycles. The mean response was then calculated for 8, approximately equally spaced 3- or 4-day intervals over the lunar cycle. The intervals were centered on the times in the monthly cycles which are illustrated in Figure 1.

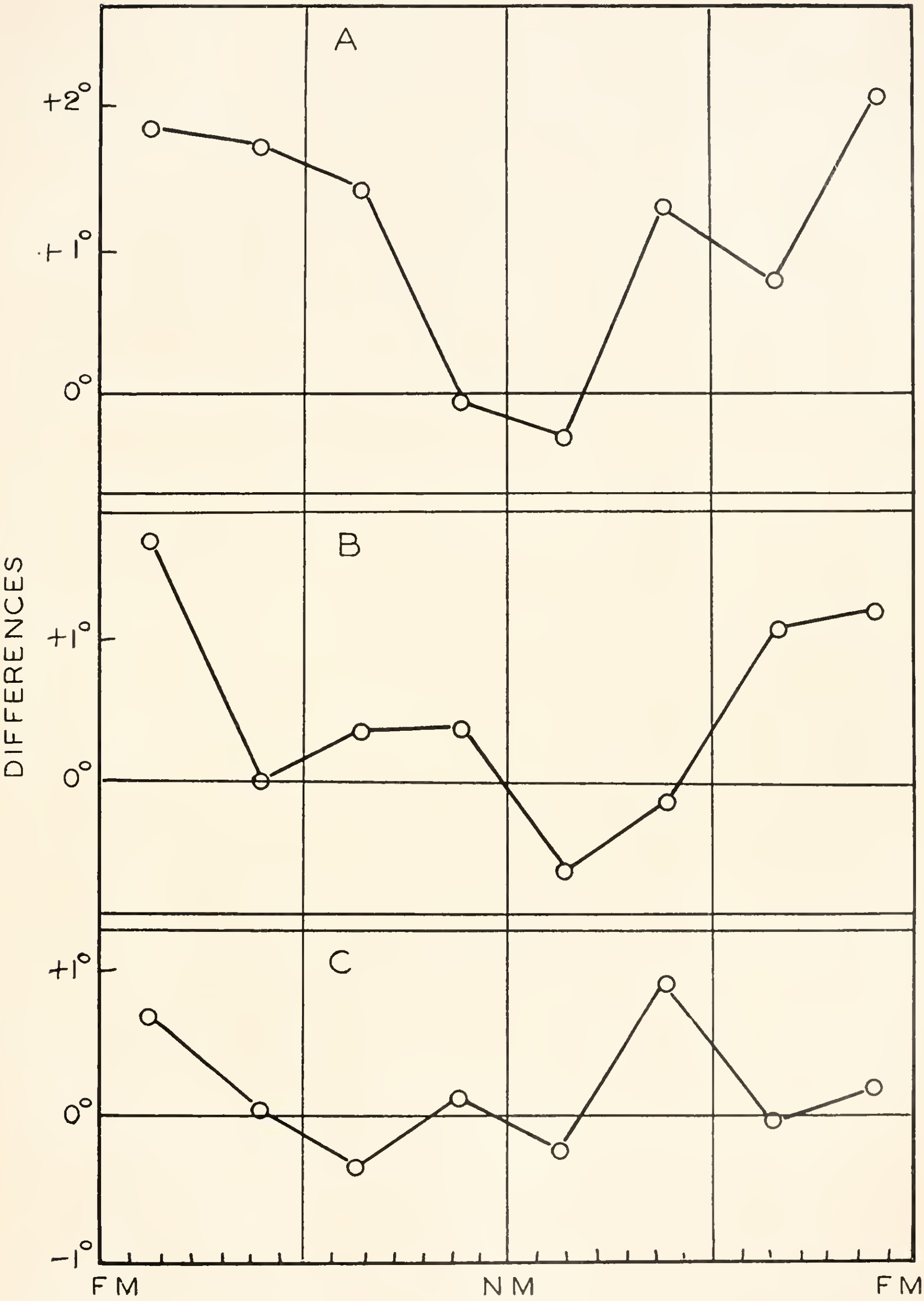

Figure 1. A. The variation in the difference between responses to the 4.0-gauss and the 0.05-gauss reversed horizontal vectors, as related to the phase of moon. B. The same difference, but for the first 15 minutes after removal of the magnets. C. The same difference, from 15 to 25 minutes after removal of the magnets.

RESULTS

In Figure 1A is plotted the mean response of the 4.0-gauss worms relative to that of the 0.05-gauss ones during the 15 minutes of exposure to the experimental fields, as a function of phase of moon. A monthly variation is clearly suggested, with a maximum difference occurring over full moon and minimum, even possibly a reversal of the difference, over new moon. The comparable variations of relative paths in the two field-strengths with phase of moon during the first 15 minutes following removal of the experimental field, and during the last 10 minutes after removal, are shown in Figures 1B and 1C, respectively. A monthly variation appears to persist for a time but to have disappeared nearly completely by 20 to 25 minutes after the field removal. The rate of the disappearance of the monthly variation in this difference strikingly coincides with the rate of loss of the general after-effect reported earlier (Brown and Park, 1965).

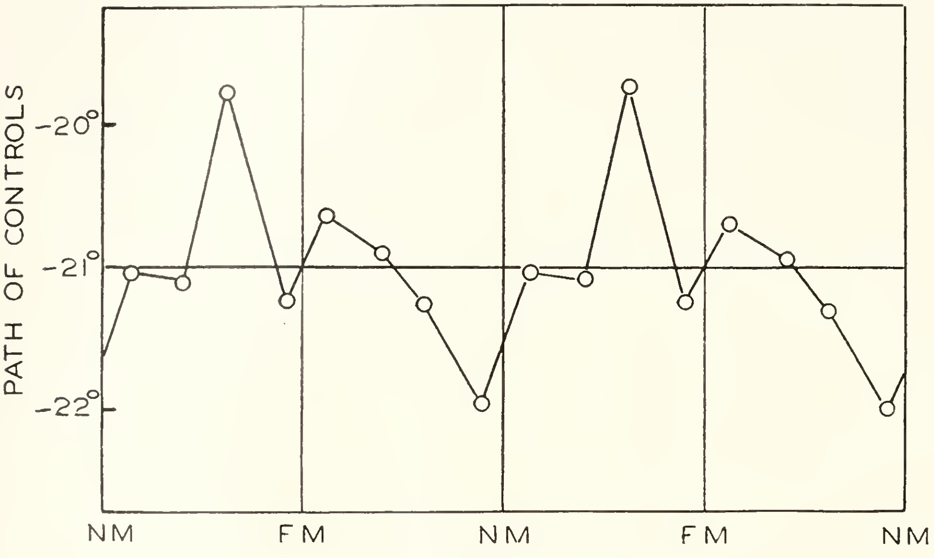

FIGURE 2. Variation in mean path of the worms (controls) with phase of moon during their initial 10-minute period while in the natural ambient geophysical field. The mean monthly cycle is repeated.

The results pertaining to general after-effects which have been described previously had suggested that during this 7-month study a mean, overall response to the two experimental field strengths occurred chiefly for the reversed 4.0-gauss one. However, evidence was advanced to indicate that the worms also displayed a very definite response to the 0.05-gauss reversed field but that the character of the response varied with time more strikingly than did response to the 4.0-gauss field, even changing in sign. The results suggested an annual variation. From the present study it became evident upon examining separately the responses to the two strengths of the magnetic fields, together with their subsequent after-effects, that response to the 0.05-gauss field varies as a function of elongation of the moon.

To learn exactly what effect the magnet might have, it was necessary first to learn the nature of the monthly variation of the initial controls. The presence and characteristics of such a rhythm in worms had previously been shown and characterized for morning hours. For the present study about 40% of the results were obtained, instead, in the afternoon. Determination of the relationship between phase of moon and means of the paths of the initial control-worms (Fig. 2) disclosed a mean monthly pattern of variation with a maximum right-turning near first quarter of the moon and maximum left-turning just prior to new moon.

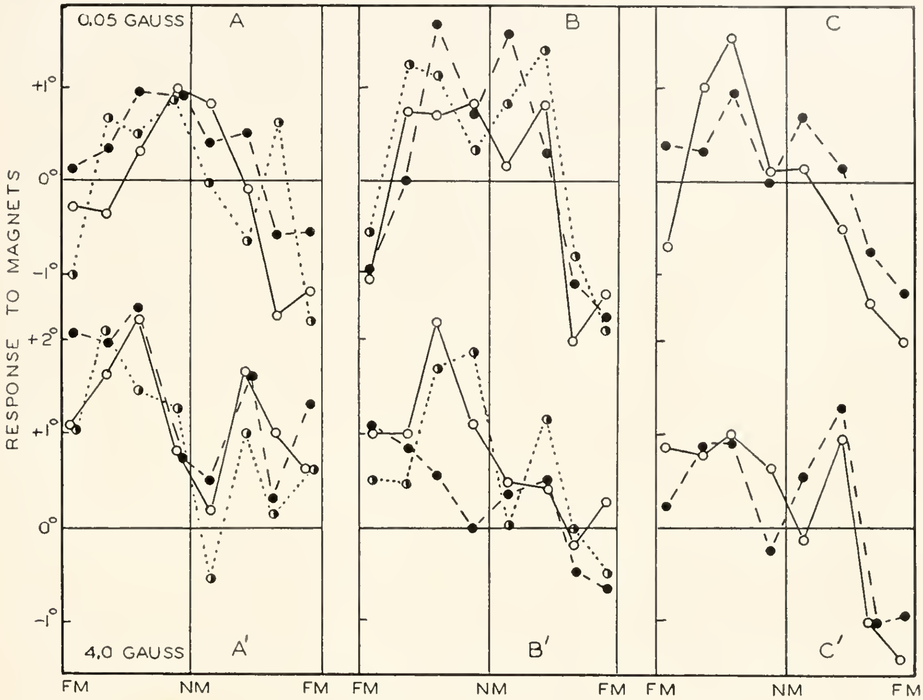

FIGURE 3. A and A'. The variation in response to the 0.05- and 4.0-gauss reversed horizontal vectors, respectively, in relation to moon phase during the first five minutes of field application (solid line), the second five minutes (dotted line) and the third five minutes (dashed line). B and B'. The corresponding relationships for the first three 5-minute intervals following removal of the magnets. C and C'. The corresponding relationships for the fourth 5-minute interval (solid line) and the fifth one (dashed line) following experimental-field removal.

The mean response to the reversed 0.05-gauss field (Fig. 3A) was an immediate gross reversal of phase of this normal monthly variation. Now, maximal *right*-turning occurred just prior to new moon. During the 15 minutes immediately subsequent to the removal of the experimental field (Fig. 3B) the amplitude of this 180° phase-shifted monthly variation slightly increased and its form became somewhat altered. Only relatively minor changes in the mean monthly pattern then seem to have ensued through the 20- and 25-minute observation times (Fig. 3C). The new cycle, 180°-shifted relative to the initial one, had persisted in an extraordinary manner.

The mean response to the reversed 4.0-gauss field was a definite and immediate displacement of the mean worm paths to the right of the controls and a slight change in the monthly pattern (Fig. 3A′). Although there was a beginning of an inversion of the monthly cycle, in that maximum right-turning occurred near third quarter of the moon, there was lacking the striking immediate, essentially complete, inversion that was observed for the response to the 0.05-gauss field. Despite, also, quite a different transient pattern from that observed for the weaker, 0.05-gauss, field during the 15 minutes immediately subsequent to magnet removal (Fig. 3B′) the increased general right-turning behavior gradually subsided and a persistently altered monthly pattern of response became evident (Fig. 3C′). The patterns of monthly variation in response witnessed during the 20- and 25-minute periods were quite similar to those observed for the corresponding periods following the removal of the 0.05-gauss reversed field, and again were essentially 180°-shifted relative to the phase of the initial control pattern.

A disappearance of all differences between the patterns of the monthly variation following the magnetic response and the pattern of the initial controls would have been expected if the shifted rhythm had gradually been lost over the 25-minute post-magnet period. And yet, the worms from both magnetic series (Fig. 3C and 3C′) now displayed, in common, a monthly pattern of difference from the initial controls which resembled astonishingly a cycle 180°-phase-shifted from that one which was present before the submission to the 15 minutes of the geographically reversed experimental magnetic field. The relationship, relative to *new* moon for persisting effect, now resembled closely the former relationship to *full* moon for the initial controls (compare Fig. 2 with Fig. 3C and 3C′).

Since the observations in these experiments had been made only during 7 or 8 daytime hours, between about 9 AM and 5 PM, the monthly rhythm of the controls could be considered as reflecting a lunar-daily variation in the orientational response of the worms, when north-directed, to the ambient geophysical field, and after magnetic field-reversal as a 180°-phase-shifted lunar-daily variation in response.

DISCUSSION

The results of this study extend our knowledge of responses of planarians to altered very weak horizontal magnetic fields to include another kind of response. This one is a phase-shifting of a lunar rhythm by an abruptly reversed horizontal vector. This response appears to be independent of vector strength at least over a range from about 1/3 to about 23 times the earth's local horizontal vector, the range in this investigation. This response appears to relate geomagnetic vector direction in some manner to phase angle in the biological lunar rhythm.

Even by the end of 25 minutes following removal of the experimental fields the relation between mean path and elongation of moon had displayed no tendency to return to its initial state. Had return occurred, the two relationships depicted in Figures 3C and 3C′ would have exhibited no characteristic monthly pattern of variation. Instead, a monthly variation of closely the same character is present for the 20- and 25-minute response patterns following the 0.05- and 4.0-gauss reversed fields. This pattern resembles, even in striking details in its form, the monthly pattern of variation of the initial controls. Even amplitude is similar. It is, however, 180° out of phase with the controls. The worms thus appear to have superimposed on their initial state a fully comparable monthly pattern with respect to full moon that as initial controls they had had with respect to new moon. In other words, the experimentally reversed magnetic fields, whether 0.05- or 4.0-gauss, had, after the very different transient states during and immediately following the reversed field, come to effect a common pattern of lunar-monthly variation in response to the natural ambient geophysical field.

In a previously reported study (Brown, 1962) a 4-gauss horizontal magnetic field parallel to the axis of N-directed planarians had abolished a distinct unimodal monthly variation which was present when the worms were in the natural ambient field. The current study indicates that a S-directed 4-gauss field, under comparable conditions, tends to convert the natural unimodal monthly variation in orientational behavior at least initially into a bimodal one (Fig. 3A′), suggestively a transitional state between the initial cycle and the 180°-shifted, essentially unimodal one which ultimately appears following return of the worms to the natural environment. At the same time this study indicates that far less conspicuous transient alterations result from application of a 0.05-gauss reversed field, judging from the general similarity between the relationships depicted in Figures 3A through C. The weaker field appeared, therefore, to be the more efficient in effecting the phase shift.

The slightly increased amplitude of the monthly variation evident immediately following the 0.05-gauss field (Fig. 3B), and the initial delay in reaching the full cycle range following exposure to the 4.0-gauss field (Fig. 3B′), suggest a hypothesis that following exposure to horizontal vectors weaker than the earth's, animals are transiently hypersensitive to the earth's vector, and following exposure to vector fields stronger than the earth's, transiently hyposensitive.

The explanation of the mean monthly variation in the response to the 4.0-gauss field when expressed as difference from response to the 0.05-gauss one, and its gradual decay following removal of the experimentally reversed horizontal magnetic vector, have now become apparent. This reflected the difference in influence on the monthly rhythm between the responses to the two strengths of experimental fields. The immediate phase-shift in response to the 0.05-gauss field, together with the initial, only partial, shift as response to the 4.0-gauss one, resulted in a monthly variation in the difference between them (Fig. 1A). This difference, though steadily diminishing during the first 15 minutes following removal of the experimental fields (Fig. 1B), did not essentially disappear until 15 to 25 minutes had elapsed (Fig. 1C). The difference had practically vanished only after both experimental groups came ultimately to stabilize with the same 180°-shifted monthly pattern.

Perhaps the most interesting and potentially most significant finding of this study is the apparent association between phase angle of a spatial vector factor, the horizontal component of terrestrial magnetism, relative to other contemporary vector forces and the phase angle of a biological rhythmic variation. Although a number of kinds of observations have suggested an existence of some common denominators for the biological clock and biological compass mechanisms, this is the first specific piece of experimental evidence that such a relationship may actually exist. While much evidence has been advanced in support of the conclusion that geographic orientation is influenced by phase-resettings of biological clocks, this is the first evidence that a biological rhythm itself can have its phase reset by altering the vector angle of any geographical field component.

The observed monthly variation in orientational tendencies of N-directed planarians is a mosaic of the activities of numerous individual members sampled from day to day from a very large population. The phase-reversal which is described here had clearly become essentially fully effected for the 0.05-gauss worms, even in time to be fully evident during the first 5-minute test assays of the worm-samples from the population over the 7-month period of study. This is hardly the type of behavior expected of an independent internal physico-chemical oscillator system as it became phase-entrained to some altered relationship. There is, obviously, no time in the phenomenon to permit conventional physiological transients. Rather, it is more plausibly like an abrupt reversal in the character of the response of the experimentally treated worm population to an external cyclic geophysical pattern. Hence, this capacity for virtually instantaneous reversal in rhythm phase appears to constitute further suggestive evidence for extrinsic timing of biological-clock systems.

SUMMARY

1. It has been shown that a 180° shift in the direction of the horizontal vector of magnetism will effect a 180° shift in the phase of the monthly rhythm in geographical orientation in planarians.

2. The shift is essentially completed immediately when the reversed field has a strength of 0.05-gauss, or about 1/3 the earth's natural horizontal vector strength.

3. When the experimentally reversed magnetic field has a strength of 4.0-gauss, more than 20 times the earth's, the complete reversal of the rhythm requires as long as 15 minutes, following the removal of the experimental fields, during which time characteristic transients intervene.

4. Implications for the biological-clock problem of this demonstrated relationship between vector direction of one geophysical component, relative to the others, and phase relationship of a fundamental biological rhythm are discussed.

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EFFECTS OF TEMPERATURE ACCLIMATION ON SOME ASPECTS OF CARBOHYDRATE METABOLISM IN DECAPOD CRUSTACEA[1]

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Temperature is one of the environmental factors to which the organism must adjust if it is to exist successfully in its habitat. In temperate-zone forms the severe temperature extremes of mid-winter and late summer may result in a shift of metabolic processes, tending to compensate for these extremes. However, tropical forms living in a relatively constant thermal environment do not have to contend with such extremes and their metabolic processes may not show a compensatory shift (Vernberg, 1962). Thus the temperature effects on an animal may be reflected in its physiology. Past investigations of temperature effects on populations have concentrated upon comparisons of rate functions such as oxygen consumption, ciliary activity, heart beat, and thermal limits of tissues and/or whole organisms (Bullock, 1955). It is to be expected that the ability to exist at an environmental temperature is expressed in the physiological and biochemical responses of the animal. The nature of these responses to temperature may vary with species or stage of the life cycle. Thus, not only is there a variation in the rate function of metabolic change with temperature adaptation, but the nature of the metabolic reaction or pathway may be altered (Ekberg, 1958; Hochachka and Hayes, 1962). The present research has been concerned with possible variations in carbohydrate metabolism with temperature acclimation. Several different species of crabs have been studied, using physiological parameters such as blood glucose, the total reducing sugar in the blood and hepato-pancreas glycogen levels. Also included was a qualitative analysis of blood carbohydrates using chromatographic techniques.

MATERIAL AND METHODS

Glucose oxidase (Huggett and Nixon, 1957) was used for the determination of blood glucose and the classic Folin-Wu method (1920) for the total reducing sugars. Glycogen was determined by the phenol-sulfuric acid method of Montgomery (1957). Chromatography of blood carbohydrates was done with ascending, descending and two-dimensional flow on Whatman #1 and #3 filter paper. Various solvents were used, with the best resolution obtained with n-butanol, ethyl alcohol, acetic acid and water in an 8:2:1:3 mixture by volume. The papers were washed in the solvent system prior to spotting the unknown. Sprays for the analysis of unknown carbohydrates included silver nitrate and sodium hydroxide,

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aniline hydrogen phthalate, iodine vapor, P-anisidine HCl and triphenyl-tetrazolium chloride. Blood samples were collected from different sites in the various species of crabs used: *Uca pugilator*, *U. minax* and *U. pugnax* were sampled in the second segment proximal to the cheliped; while *Callinectes sapidus* were sampled from the sinus at the base of the fifth pereiopod and for *Cancer irroratus*, *Libinia emarginata*, *Panopeus herbstii* and *Menippe mercenaria*, directly from the heart. Males only were used for analysis to reduce the possible effects of the hormonal factors associated with the reproductive cycle in the female (Dean and Vernberg, 1964). For chromatography, the blood was deproteinized by heating in a boiling-water bath for one minute and the supernatant fraction obtained after centrifugation was desalted (Dowex AG-501-X8); the resulting effluent was taken to dryness, dissolved in water to give a concentration equivalent to about 20 micrograms per microliter of dried material and standards were run with each unknown (McWhinnie and Saller, 1960). R_f and R_g values were calculated for comparison with values obtained by other workers. In acclimation experiments, other than the chromatography, *Uca pugilator* were used. These animals were acclimated to a given temperature for a minimum of three weeks. They were kept under a uniform 14 hours light and 10 hours dark photoperiod and the water was main-

CHROMATOGRAPHIC PRESENCE (+) OR ABSENCE (-) OF CARBOHYDRATES IN BLOOD

	Maltotetraose Maltotriose Maltose Glucose	Galactose	Mannose	Fucose	Galactan Derivative
Callinectes sapidus	+	+	±	-	+
Cancer irroratus	+	+	-	-	+
Libinia emarginata	+	+	±	±	+
Menippe mercenaria	+	+	+	+	+
Panopeus herbstii	+	+	-	-	+
Uca minax (Early Spring)	+	+	-	-	+
Uca minax (Autumn)	+	+	±	±	+
Uca minax (10°), (18°), (28°)	+	+	-	-	+
Uca pugilator (4°), (18°), (30°)	+	+	-	-	+
Uca pugilator (Early Spring)	+	+	+	-	+
Uca pugilator (Autumn)	+	+	+	-	+
Cancer magister[1] and *Hemigrapsus nudus*	+	±	-	±	-
Orconectes virilis[2]					

1 Meenakshi and Scheer

2 McWhinnie and Saller

TABLE I

tained at a constant salinity of 30‰ and changed every 36 hours on an 8 AM, 8 PM, 8 AM schedule. The diet consisted of Clark's fish pellets. This maintenance procedure resulted in a very low mortality in the laboratory animals. For the eyestalk experiments, the eyestalks were removed at their base and the wound closed with a cold cauterizer.

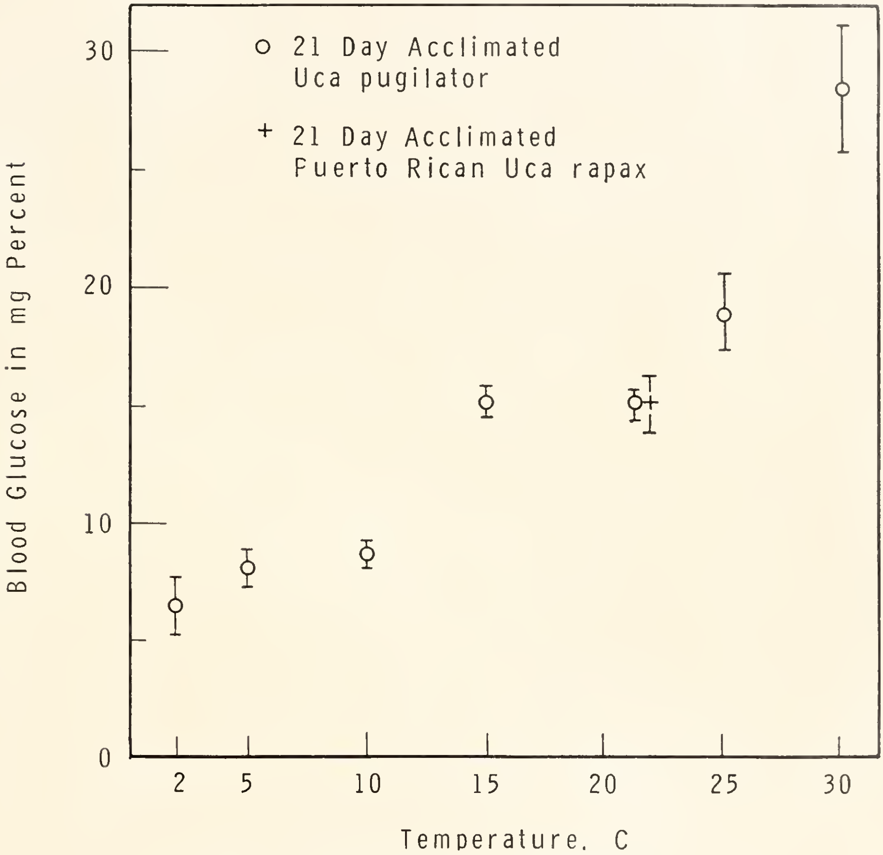

FIGURE 1. Blood glucose in acclimated crabs.

RESULTS AND DISCUSSION

The qualitative chromatography of blood carbohydrates, as seen in Table I, in seven species of crabs gave similar results. All species have maltotetraose, maltotriose, maltose, galactose, glucose and a galactan derivative. Some differences occur in the appearance of mannose and fucose in some species. Mannose is present in *Menippe* and early-spring *Uca pugilator* and possibly in *Libinia*, autumn *Uca minax* and *Callinectes*. Fucose is definitely present in *Menippe* and possibly in *Libinia* and autumn *Uca minax*. Glucose-6-phosphate is known to be present in the blood of these crabs from other work done in this laboratory. These results compare favorably with those obtained by Hu (1958) for the shore crab, *Hemigrapsus nudus* and *Cancer,* and the work of McWhinnie and Saller (1960) on the fresh-water crayfish, *Orconectes.* Fairbairn (1958), using disc chromatography, has demonstrated trehalose in the tissues of several crustaceans. Using colorimetric techniques, we detected trace amounts of trehalose in the blood of several species and a higher amount was found in the blood of *Libinia.* However, these results are quite variable. Samples of blood of three species of *Uca* acclimated to different temperatures have been analyzed by chromatography. No qualitative differences could be seen in the blood carbohydrates.

Results of blood glucose and total reducing sugar levels of crabs acclimated to different temperatures would indicate that the concentration of glucose in blood is depressed at the lower temperatures (Fig. 1). A minimum of 15 individual samples was used in the determination of each point, and the figures show the mean value and standard error. Blood glucose is usually 20–25% of the total reducing sugar value of the blood. This ratio does not seem to vary significantly with

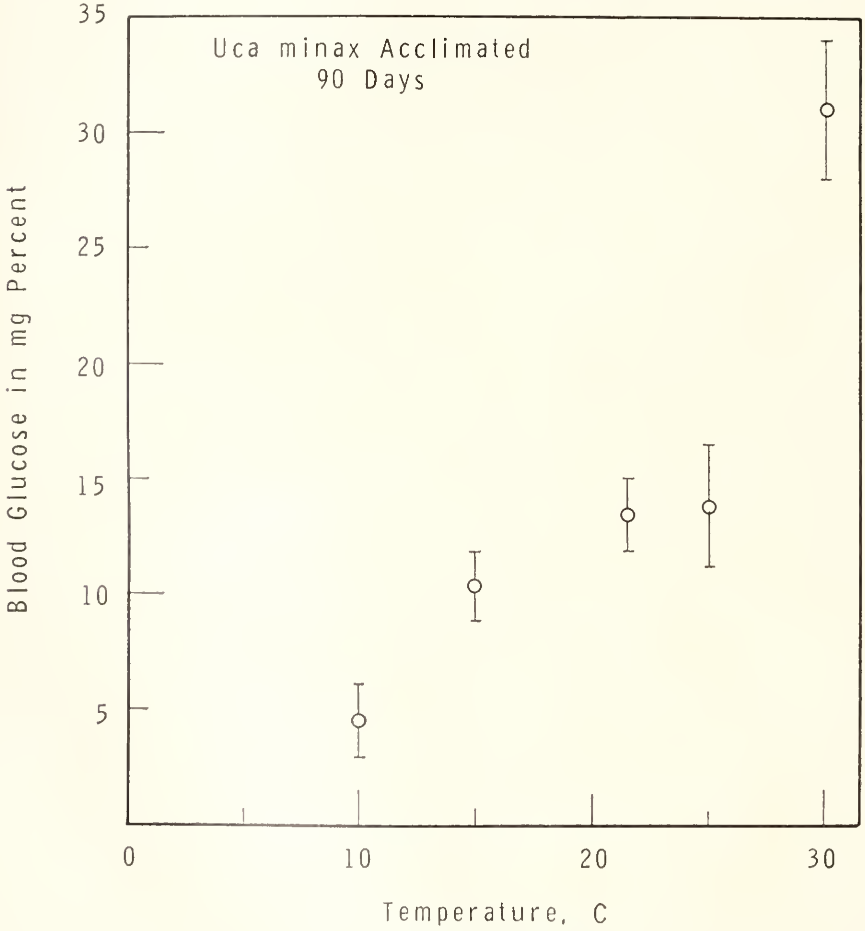

FIGURE 2. Blood glucose in acclimated crabs.

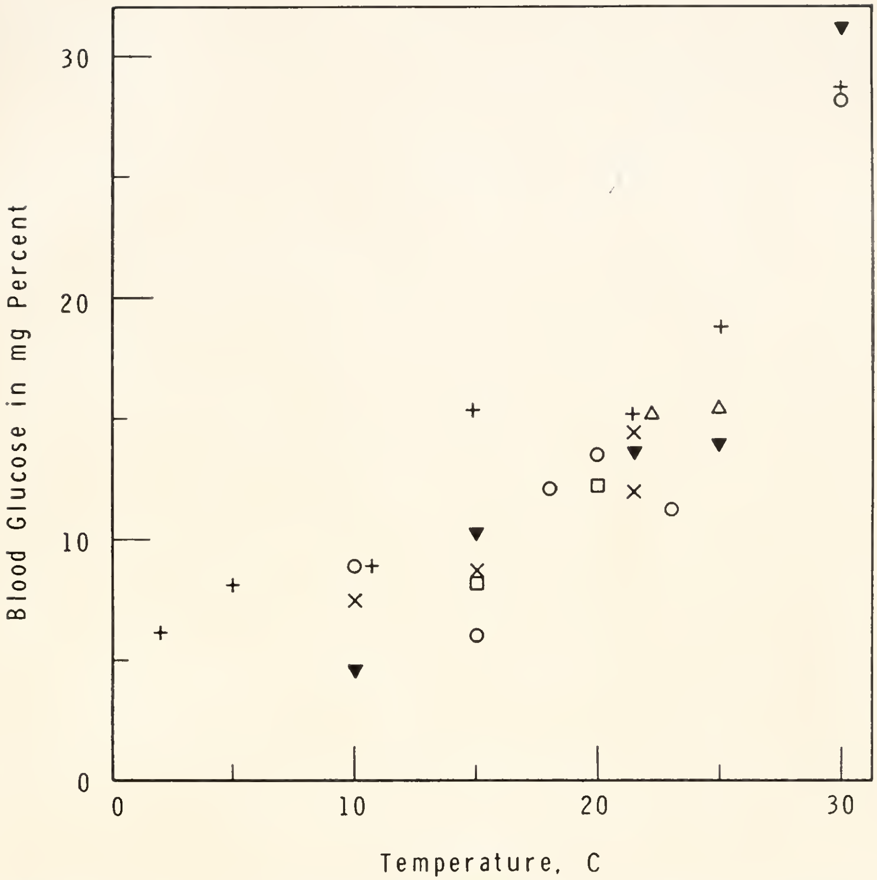

FIGURE 3. Blood glucose in crabs (X—*U. pugilator*—field animals, +—*U. pugilator*—21 day acclimation, ○—*U. minax*—21 day acclimation, ▼—*U. minax*—90 day acclimation, △—*U. rapax*, □—*U. pugnax*).

acclimation. Also, a tropical species (*Uca rapax*), acclimated at the same temperature as temperate species, has similar blood glucose values. Apparently laboratory acclimation had results similar to natural field conditions because 2° laboratory-acclimated *U. pugilator* had a low blood glucose value, as did the newly emerged crabs in early March. Short periods of acclimation to higher temperatures resulted in a higher blood glucose level, and long-term acclimation to high temperature, as in *U. minax*, showed an even more marked increase. However, *U. minax* shows much the same response as *U. pugilator* (Fig. 2). Long-term acclimation, in this case three months, resulted in a trend to temperature-dependent blood glucose values. The level for field animals emerging early in the spring and acclimating animals followed the patterns seen in *U. pugilator*.

Uca pugnax, a temperate-zone form, fits the range of blood glucose values for the other species (Fig. 3) and *U. rapax*, which is a tropical species, is also in the same general range. Thus, it may be seen that there is a general trend to low blood glucose at low temperature and an upward trend at higher temperatures. The data indicate a plateau for blood glucose around the optimum temperature range of the animal, which is a pattern similar to that seen in respiration experiments (Vernberg, 1959).

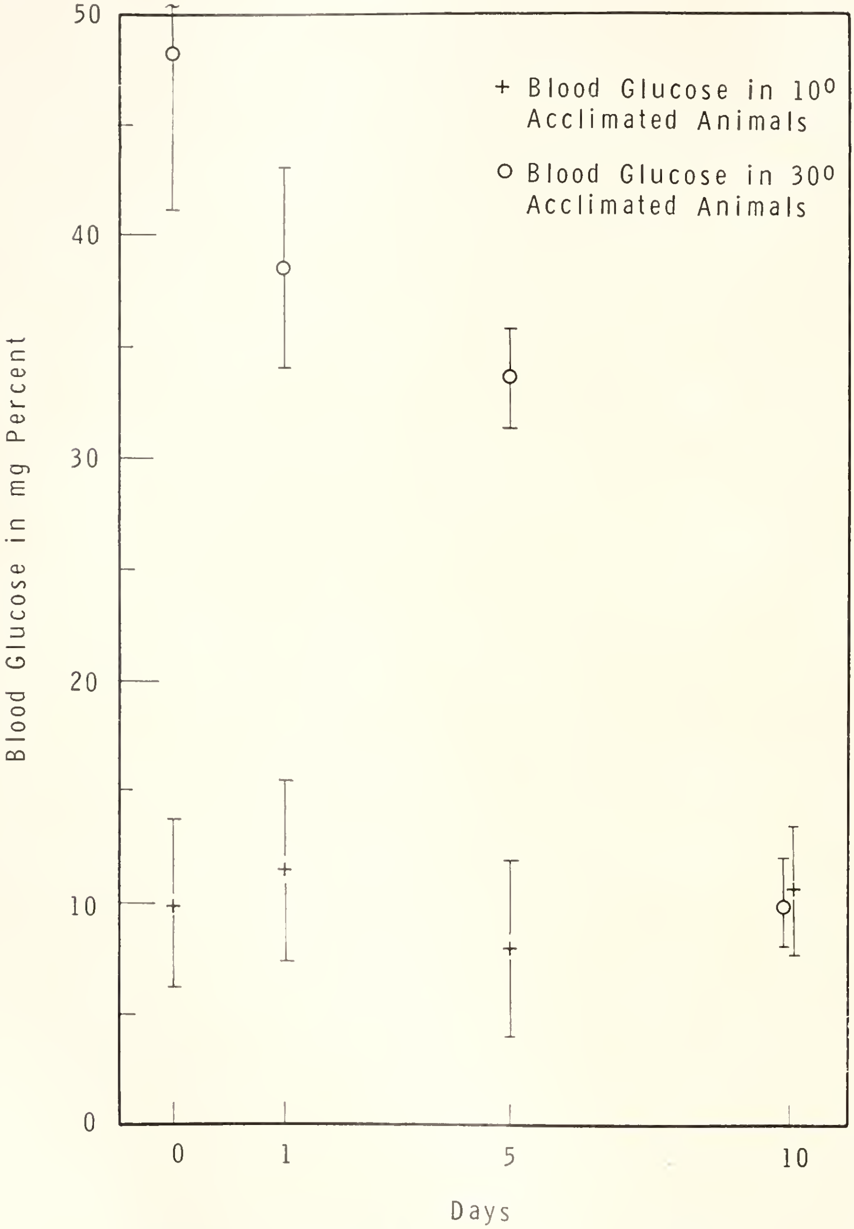

FIGURE 4. Effect of fasting on blood glucose in *U. pugilator*.

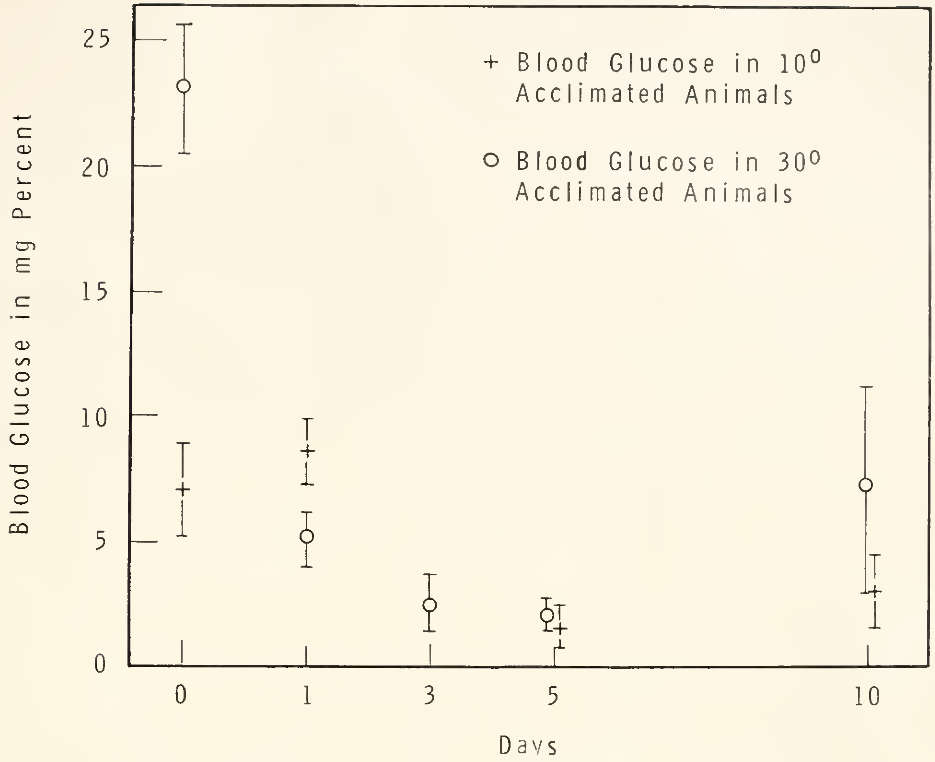

FIGURE 5. Effect of eyestalk removal on blood glucose in *U. pugilator*.

Qualitatively there is no major change in blood carbohydrates with acclimation. However, there may be quantitative differences, as shown by the lower blood glucose values obtained with animals acclimated to low temperatures.

We were interested also in the effects of diet and hormones in relationship to the carbohydrate metabolism of the animal. Crabs were well acclimated for 21 days at 10° and 30°. They were then fasted and sampled on days 0, 1, 5 and 10. Unlike *Libinia*, which shows no change in blood sugars with fasting or eyestalk removal (Kleinholz and Little, 1949), it may be seen in Figure 4 that the blood glucose in the fasting 30° *U. pugilator* decreased consistently with time while the fasted 10° animals were fairly constant. The hepato-pancreas glycogen levels followed this same pattern.

Eyestalk removal during the intermolt stage will induce molting in the crab except at lower temperatures where temperature acts as a molt inhibitor (Passano, 1960). During ecdysis, a period of high physiological activity, several dramatic changes in the carbohydrate metabolism occur. To induce molting, eyestalks were removed from 10° and 30° acclimated *U. pugilator* fed *ad libitum*. Samples were taken on days 0, 1, 3, 5 and 10. Under these conditions, which initiate the molt sequence of events, there is an extremely rapid drop in the blood glucose level of the 30° animals (Fig. 5). This remains at a low level for a period of time and then begins a slow increase. However, the 10° animals did not show this change in blood glucose level, did not molt or initiate proecdysis, and had a high mortality rate. There is no significant change in the hepato-pancreas glycogen values in the 10° animals but the 30° crabs showed a typical buildup in hepato-pancreas glycogen as molt approaches, and rapid decline with ecdysis.

The authors are grateful to Miss Rosemary McCarthy for her fine technical assistance and the J. R. Clark Co., Salt Lake City, Utah for supplying the diet.

SUMMARY AND CONCLUSIONS

The preceding work on carbohydrate metabolism in *Uca* would suggest the following:

First, with temperature acclimation there is no qualitative shift in carbohydrate metabolic pathways, but rather there may be quantitative variations. Second, at low temperature the animal reduces its energy output to a minimal level. This may be related to the energy demands of the molt cycle. It would seem that even though sufficient carbohydrate reserves are present at low temperature, there may be variations in hormone levels which would affect the molt cycle. These physiological characteristics correlate well with field observations and the general ecology of the fiddler crab. However, generalities cannot be made for all Crustacea as there are obvious differences between genera.

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PINOCYTOSIS OF PROTEINS BY OYSTER LEUCOCYTES[1,2,3,4]

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The term "pinocytosis" (cell drinking) was first introduced by Lewis (1931). This same phenomenon, however, was observed earlier by Metchnikoff (1901) in mammalian leucocytes in an aseptic exudate induced by a 10% gelatin solution, and also by Edwards (1925) in amoebae when certain simple salts were added to their culture medium. Later Mast and Doyle (1934) found that, in the presence of albumin or calcium gluconate solutions, *Amoeba proteus* and a number of related species also display pinocytic activities. After the publication of these papers, pinocytosis was virtually unheard of for nearly 20 years. Recently this old subject has been investigated with renewed vigor by using new techniques: *e.g.,* electron microscopy, fluorescence microscopy, interference microscopy, radioisotope and fluorescent dye labelling, and serological methods. A brief review of the literature reveals that pinocytosis has been demonstrated for mammalian cells, in tissue cultures, such as polymorphs (Bessis and Bricka, 1952), macrophages (Lewis, 1931), erythroblasts, normoblasts, reticulocytes and certain pathological erythrocytes (Bessis and Breton-Gorius, 1957), sarcoma cells (Lewis, 1937), ascites tumor cells (Easty, Ledoux and Ambrose, 1956) and HeLa cells (Rose, 1955); for protozoa such as *A. proteus* (Lewis, 1937), *Chaos chaos* (Holter and Marshall, 1954; Brandt, 1958), *Plasmodium lophurae* and *P. berghei* (Rudzinska and Trager, 1957, 1959); and for blood elements of invertebrates, *e.g.,* elaiocytes of the coelomic fluid of echinoderms (Holter, 1959) and phagocytes of planarians (Rosenbaum and Rolon, 1960).

Oyster leucocytes are known to ingest a variety of particulate materials (Yonge, 1926; Takatsuki, 1934; Stauber, 1950; Tripp, 1958a, 1958b, 1960; Feng, 1962). In the present study, the *in vivo* and *in vitro* uptake of proteins by oyster leucocytes was demonstrated by using serological techniques and fluorescence microscopy.

MATERIALS AND METHODS

1. *Oysters, aquaria* and *sea water*

The oysters and sea water used in this study were collected from the Navesink River, near Red Bank and the Shrewsbury River at Highlands, New Jersey, re-

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[2] This investigation was supported in whole by Public Health Service Research Grant AI-00781, from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health.

[3] In the oyster the terms *amebocyte, leucocyte,* and *phagocyte* are used interchangeably, as suggested by Stauber (1950).

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spectively. Pyrex battery jars, measuring 25 × 25 cm., were used as aquaria to hold oysters. The oysters were kept in sea water of 20‰ throughout the experiment. Constant aeration was maintained in each aquarium. Water was changed at least twice daily.

2. *Preparation of oysters for injection and bleeding*

Exposing the heart for injection and bleeding was achieved by following the established procedure of Feng (1965).

3. *Preparation, injection, and detection of inocula*

a. Bovine hemoglobin solution

A 5% crystalline bovine hemoglobin solution was prepared in a diluent of oyster plasma which was drawn and pooled from 10 oysters. Desired amounts of this solution were injected into oysters *via* the ventricular route.

The reduction of bovine hemoglobin from the blood stream by the oyster was followed colorimetrically by sampling the heart blood at appropriate intervals over a period of 154 minutes. The presence of bovine hemoglobin within the leucocytes after injection was detected visually by the pink coloration of the washed sedimented blood cells.

b. Diphtheria antitoxin

Horse antibody to diphtheria toxin, kindly supplied by Dr. R. J. DeFalco, Director of the Serological Museum, Rutgers, The State University, was injected into 10 oysters in volumes of 0.2 ml. per animal (3125 units per ml. of antiserum). Pooled blood samples were taken from the oysters in the amount of 0.2 ml. per oyster at 10 minutes, 1, 2, 4 and 8 hours. Oyster leucocytes were separated from the plasma by centrifugation and washed in several changes of sea water. The cells were ruptured by grinding them with fine sand and the extract was reconstituted to 2.0 ml. with 0.85% saline. The sand was removed by centrifugation.

The presence of the diphtheria antiserum in both the plasma and leucocyte saline extract was detected by reacting them with diphtheria toxoid, using a modified procedure of Ramon titration. The procedure of ordinary Ramon titration (Boyd, 1956) consists of mixing constant dilutions of toxoid with varying dilutions of antiserum or *vice versa*. The amount of toxoid which gives most rapid flocculation with one standard unit of antiserum is first determined. The amount is designated as the Lf unit. Then unknown antisera can be titrated against this standardized toxoid. The time required for the first flocculent precipitate to occur is referred to as Kf, or flocculation time. The modified procedure devised for this work is carried out under the condition that the Lf units of both the toxoid and antiserum are known. When materials containing unknown units of toxoid or antiserum are added to the above system, the flocculation time will change accordingly.

Perhaps the points made above are best illustrated in the following example:

Tube No.	1	2	3	4
Toxoid (50 Lf/ml.)	0.5 ml.	0.5 ml.	0.5 ml.	0.5 ml.
Antiserum (312.5 units/ml.)	0.06 ml.	0.08 ml.	0.10 ml.	0.12 ml.
Kf (minutes)		35		

Tube No. 2 is the first one to show the flocculent precipitate after 35 minutes incubation at 40° C. If 0.5 ml. oyster plasma containing an unknown amount of antiserum was mixed with the toxoid in each of the above four tubes and then the usual amounts of antiserum added, there could be the following possible consequences: the Kf of Tube No. 2 could be prolonged, shortened or unchanged depending on the amount of antiserum contained in the oyster plasma; or Tube No. 3 could be the first one to show the flocculent precipitate. If the latter case took place, the unknown might be solved as follows: $50 \text{ Lf} \times 0.5 + X = 312.5 \times 0.10$, where X represents the unknown Lf units contained in 0.5 ml. of oyster plasma.

c. Rhodamine-labelled proteins

Conjugation of crystalline human gamma globulin, albumin fraction V and *Limulus* serum with Lissamine Rb 200 was carried out as specified by Chadwick, McEntegart and Nairn (1958). The conjugated proteins were brought to pH 7.8 and normal tonicity by dialyzing overnight against filtered sea water (20‰). The final concentration of these solutions was 2.5 gm. per 100 ml.

The fluorescence equipment used in the present study is manufactured by Reichert. It consists of a regular monocular microscope, with a high pressure mercury arc (HBO 200) as source of excitation. When viewed under the UV microscope with Schott BG-12 (3 mm.) and Corning-5840 as primary filters and Eastman Kodak WA-15 as secondary filter, the rhodamine conjugates exhibit a brilliant orange fluorescence which is readily distinguished from the blue-green intrinsic fluorescence of oyster leucocytes and other tissues.

RESULTS

1. *Bovine hemoglobin*

Three oysters (A, B and C) were injected with 0.3, 0.2 and 0.15 ml. of a 5% bovine hemoglobin solution, respectively. The different rates of disappearance of hemoglobin from the heart blood shown by the three oysters reflect differences in the amount of inoculum received, since the three oysters were comparable in size (Fig. 1). According to Figure 1, 130, 63 and 48 minutes were required to reduce 50% of the injected hemoglobin by oysters A, B and C, respectively.

It was noticed that nearly all samples of oyster whole blood taken 30 minutes after injection contained some pink leucocytes. The leucocytes retained their pink coloration even after several gentle washings in filtered sea water.

2. *Diphtheria antitoxin*

Since the ionic concentration and other constituents of oyster plasma and leucocyte saline extract are probably quite different from those of mammalian sera, a series of controls designed to test the effects of oyster plasma on the toxoid-antitoxin system was first initiated. The result (Table I) indicates that adding 0.5 ml. of 0.85% saline to Control A increases the Kf almost 71% (line B). Addition of either oyster plasma or leucocytes saline extract to the toxoid-antitoxin system greatly retards the reaction; in certain instances the Kf is 2 to 5 times longer than the control (compare B with C; E with F and G). Superficially, the inhibitory effect of oyster plasma appears to be stronger than that of leucocyte saline extract. The importance of this difference will remain unanswered until information concerning the precise protein content, ionic composition and other constituents in a unit volume of oyster plasma and leucocyte saline extract becomes available. The data also suggest that the Kf is a function of the concentration of reagents. The toxoid of Controls B, C and D contains 25 Lf units, while the antiserum of Control E, F and G is 125 Lf units. Consequently, the Kf for the latter group is 3 to 12 times shorter than that of the former group. Hence, wherever a considerable shortening of Kf occurs in the experimental group, as contrasted with the proper control, it is implied that this shortening of Kf is probably due to the pres-

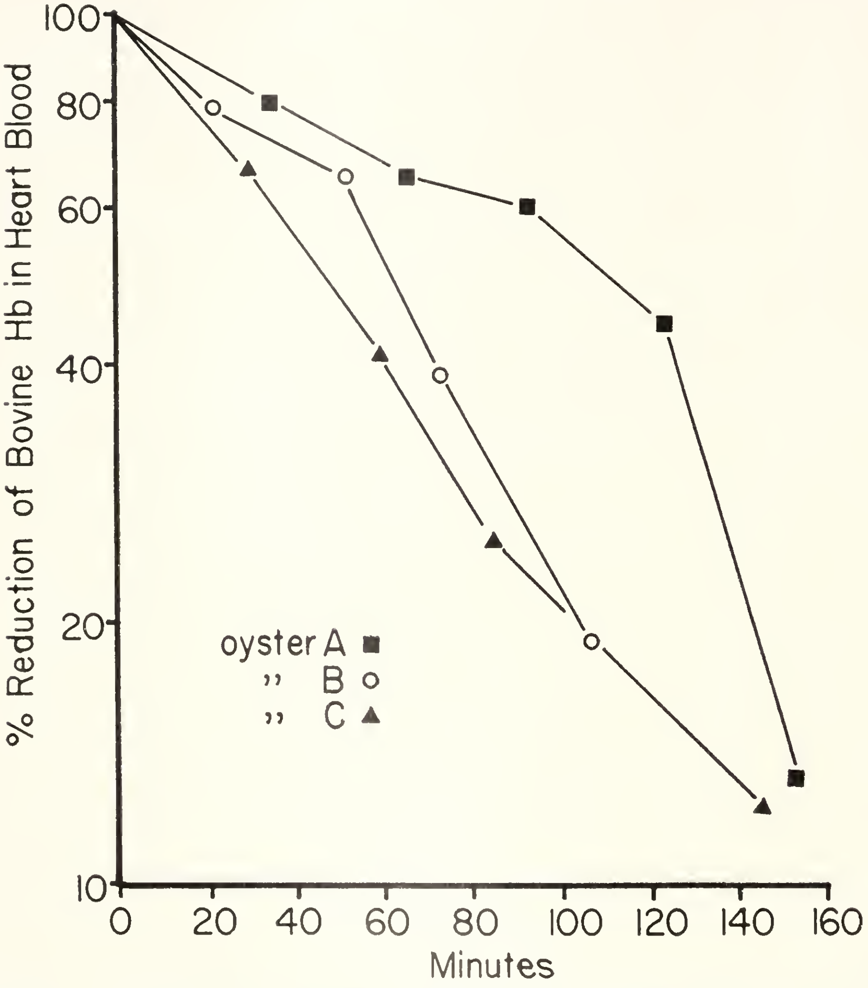

FIGURE 1. The reduction of intracardially injected bovine hemoglobin from the heart blood of three oysters.

ence of an unknown amount of Lf units in the oyster plasma and leucocyte saline extract.

Based upon the above considerations, it is found that the presence of diphtheria antiserum in the oyster plasma could be detected 10 minutes to four hours after the injection, while in the leucocyte saline extract it probably lasted two hours. However, the subsequent negative results do not necessarily suggest that the injected material is degraded but may merely indicate that the concentration of this material becomes too low to be detected by this procedure.

Table I

The detection of diphtheria antiserum (horse) in oyster plasma and leucocyte saline extract, expressed in terms of flocculation time (Kf) by using a modified Ramon titration procedure

System	Flocculation time (Kf) in minutes						Remarks
	Control	10 min.	1 hr.	2 hr.	4 hr.	8 hr.	
		Experimental samples					
A. T-A	35	—	—	—	—	—	T(constant) = 25 Lf/0.5 ml. A(variable) = 312.5 Lf/ml.
B. T-S-A	60	—	—	—	—	—	
C. T-Op-A	120	—	—	53	65	*	
D. T-O1-A	68	—	—	48	71	65	
E. A-S-T	5	—	—	—	—	—	A(constant) = 125 Lf/0.04 ml. T(variable) = 300 Lf/ml.
F. A-Op-T	25	5	30	—	—	—	
G. A-O1-T	20	**	8	—	—	—	

T, A, S, Op and O1 represent diphtheria toxoid, diphtheria antiserum, saline, oyster plasma and oyster leucocyte saline extract, respectively. The saline used is a solution of 0.85% NaCl.

* No reaction was noticed after three hours.

** Sample lost.

3. *Rhodamine-labelled proteins*

a. The effect of concentration on the uptake of rhodamine-labelled human gamma globulin by oyster leucocytes

Four rhodamine-labelled human gamma globulin solutions: 2.5, 0.25, 0.025 and 0.0025 gm.%, were used in the experiment. One drop of the above solutions was mixed with four drops of oyster blood on a slide. The drops of protein solution and oyster blood were delivered by a tuberculin syringe with a 30-gauge needle. Thus, on the slide the leucocytes were exposed to rhodamine-labelled human gamma globulin solutions with the following final concentrations: 0.5, 0.05, 0.005 and 0.0005 gm.%. After the leucocytes were exposed to the solution for a specified period (5, 15, . . . 60 minutes), the excess protein solution was removed by several gentle washings with filtered sea water. The preparation was sealed with a Vaselined coverglass and viewed under the UV microscope. Each sample consisted of three such preparations. The number of leucocytes containing orange-red fluorescent dots in at least 100 leucocytes was used to calculate the per cent pinocytosis by the leucocytes. Both experiments were carried out at 23° C.

The results shown in Figure 2 suggest that in both experiments the leucocytes require longer time to take up protein from a less concentrated rhodamine-labelled human gamma globulin solution than from a more concentrated solution. It is noticed that there are differences between experiments but the general order of concentration effect on the rate of uptake of the protein solution by leucocytes is consistent. For instance, to reach the level of 20% pinocytosis by the leucocytes

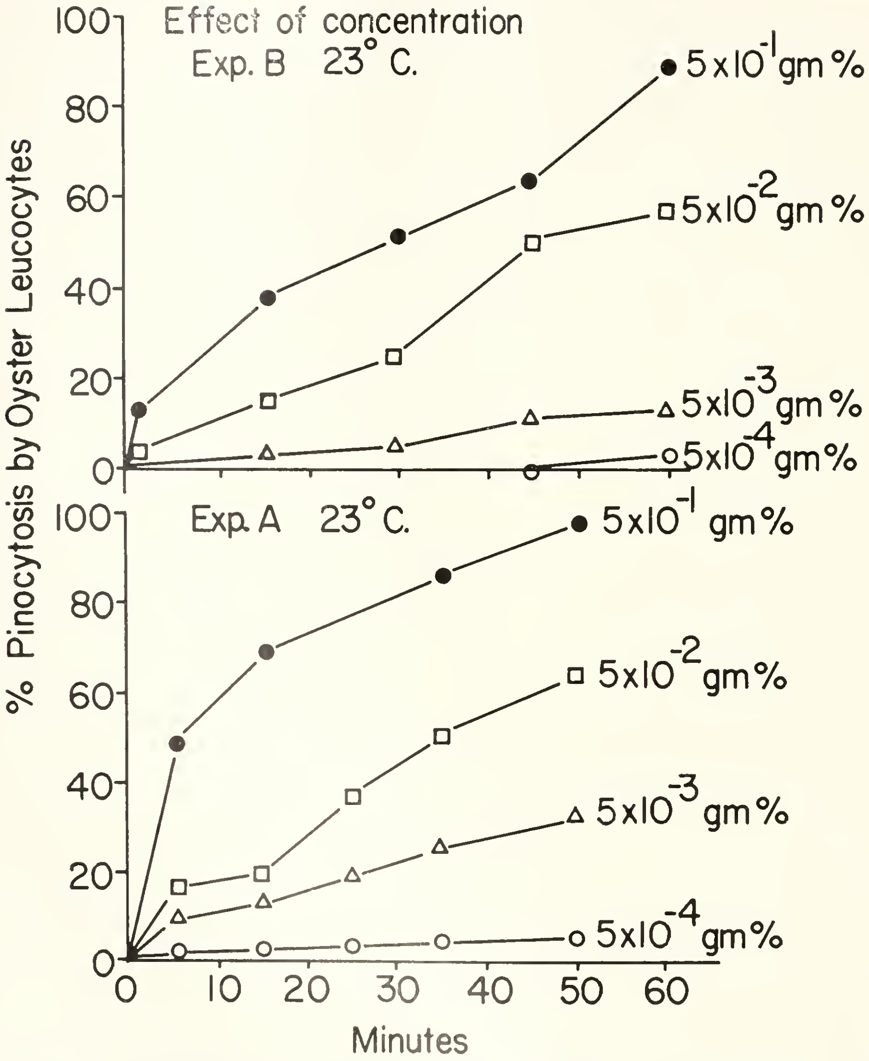

FIGURE 2. The effect of concentration and time of exposure on the pinocystosis of rhodamine-labelled human gamma globulin by oyster leucocytes. Each point on the graph represents the median of three samples.

in Experiment A, 2, 15 and 25 minutes are required for 0.5, 0.05 and 0.005 gm.%, respectively; for 0.0005 gm.%, less than 5% of the leucocytes showed pinocytosis at the end of 50 minutes. In Experiment B for comparable concentrations of protein solution, the relative rate of uptake by the leucocytes is in general lower than that of Experiment A, *i.e.*, 5 and 22 minutes for 0.5 and 0.05 gm.%, respectively. Also at the end of one hour less than 15% and 5% of the leucocytes showed pinocytosis in 0.005 and 0.0005 gm.%. Efforts were made to render conditions as comparable as possible in the two experiments. However, the number of leucocytes per drop of oyster blood as delivered by the tuberculin syringe with a 30-gauge needle was not determined for the two experiments. It is suspected that some of the differences in the rate of uptake in the two experiments might result from unequal numbers of leucocytes in the drops of oyster blood used.

b. The effect of temperature on the uptake of rhodamine-labelled human gamma globulin by oyster leucocytes

Two experiments were performed at 10° and 24° C., respectively. In conducting the experiment at 24° C., the experimental procedure was similar to that of Experiment A and B in the above study. For the experiment carried out at 10° C., special procedures were followed in order to maintain the leucocytes, inoculum and instruments used in this experiment at the same temperature. The temperature of a refrigerator was adjusted so that the temperature was 10° C. on the bottom shelf where the inoculum and a tuberculin syringe with 30-gauge needle were stored overnight. Fresh oyster heart blood was obtained by bleeding the animal at room temperature. Four drops of oyster blood were placed on each of the 15 slides. They were immediately stored in the refrigerator in a moist chamber to prevent excessive evaporation. At the end of 30 minutes, one drop of 2.5 gm.% rhodamine-labelled human gamma globulin was added to each of the 15 leucocyte preparations. At each predesignated time interval, three leucocyte preparations were removed from the refrigerator, washed, sealed and examined as described for Experiments A and B reported above.

Figure 3 indicates that the uptake of the rhodamine-labelled human gamma globulin (0.5 gm.%) by the leucocyte is considerably faster at 24° than at 10° C. In order to attain the level at which 50% of the leucocytes show pinocytosis, 12 and 28 minutes were required for the leucocytes held at 24° and 10° C., respectively.

c. Distribution of rhodamine-labelled proteins in the tissue of oysters

Three groups of oysters (A, B and C), each consisting of 8 oysters, were used for the injection of rhodamine-labelled human albumin, human gamma globulin and *Limulus* serum protein, respectively. Each oyster was injected with 0.2 to 0.4 ml. of the above labelled proteins. A fourth group (D) with 8 uninjected oysters served as a control. The experiment was performed with the temperature ranging from 18° to 20° C. Sampling was made at the following intervals: 15 minutes, 1, 2 and 4 hours, and 1, 2, 4 and 6 days. At the above intervals, four oysters, one from each of the four groups, were sacrificed by removing the shell. The whole oyster was cut into three pieces through the following regions: oral hood, visceral mass and adductor muscle. These pieces of oysters were wrapped separately with aluminum foil, dropped into liquid nitrogen for quick freezing and stored in the refrigerator at −25° C., to be sectioned with a freezing microtome. The sections placed on glass slides were fixed briefly with 10% formalin in sea water. They were then dehydrated in three changes of dioxane of four minutes each and mounted in non-fluorescent medium. The distribution of fluorescent proteins in tissues was ascertained by noting (1) the presence or absence of inoculated substances free or pinocytosed in selected lumina (blood vessels, intestine, digestive diverticulum, etc.), and (2) migration of protein-laden leucocytes through various epithelia.

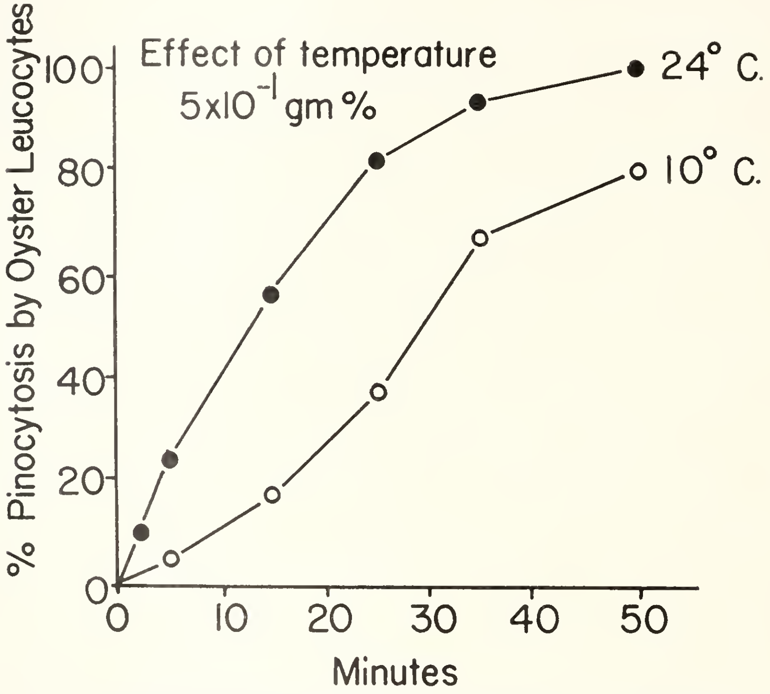

FIGURE 3. The effect of temperature and time of exposure on the pinocytosis of rhodamine-labelled human gamma globulin by oyster leucocytes. Each point on the graph represents the median of three samples.

Regardless of the fact that the inocula consisted of three different protein solutions, no outstanding differences were found in their distribution or in the subsequent elimination of the inoculated materials from the oyster. Thus, unless otherwise noted, the following results apply to all three inocula. The blood in the arterial and venous systems (anterior aorta, posterior aorta leading to the adductor muscle, blood spaces in the muscle, small arteries in the visceral mass, blood spaces immediately adjacent to the stomach, intestine, style sac and rectum, sinuses in the mantle, medial gill axis vein and lateral palliobranchial veins) appeared to be filled with the labelled proteins 15 minutes after intracardial injection. At no time during the experiment were labelled proteins detected in the circumpallial arteries.

In the subsequent samples, the general pattern of distribution of the labelled protein was essentially similar to that of the 15-minute sample described above. The only noticeable change was that the amount of free labelled proteins in the lumina of blood vessels and spaces decreased with time; this is indicated by the gradual reduction of orange-red fluorescence in these areas. Concurrently with the above observation, it was noticed that some of the labelled proteins outlined the blood spaces as though adsorbed to the cells delimiting these spaces. The labelled materials were found to be pinocytosed by leukocytes in the peri-intestinal region within one hour post-injection. Migration of the protein-laden leucocytes across the arterial wall was not observed in all samples. However, migration of protein-laden leucocytes across the epithelia of stomach, intestine, rectum, digestive diverticula and mantle facing the palp was first noticed 15 minutes after injection of labelled human gamma globulin. This process did not commence in the oysters inoculated with labelled human albumin and *Limulus* serum protein until two hours post-injection. Rejecta and dejecta collected from the aquarium 24 hours post-injection showed numerous leucocytes containing rhodamine-labelled proteins. This observation constitutes further evidence for the elimination of some of the injected protein solutions. Epithelial linings of mantle facing the shell and promyal chamber were only occasionally used by the protein-laden leucocytes as exits. No protein-laden leucocytes were observed to traverse the wall of gonoducts and nephridial tubules. Protein-laden leucocytes were also seen in the external lining of the heart and the parietal pericardium. Although protein-laden leucocytes were only very rarely seen in the lumina of stomach, intestine, digestive diverticula and rectum, the observation that large numbers of protein-laden cells were found in the dejecta has led the writer to believe that the rare occurrence of such cells in these regions might be due to the fast emptying time of the digestive tract and the concentration represented by the formation of dejecta.

Discussion

The distribution and the sites of elimination of the injected rhodamine-labelled proteins in oyster tissues in general do not differ significantly from those obtained by the injection of other particulate materials (Stauber, 1950; Tripp, 1958a, 1958b, 1960). Only the wide distribution of the labelled proteins and the earlier commencement of migration of protein-laden leucocytes were in contrast with the findings of Stauber and Tripp. For example, migrations of protein-laden cells were first encountered in the epithelia of stomach, intestine and digestive diverticula 15 minutes to two hours post-injection (18° to 20° C.), whereas the process in the same area was observed 8 days post-injection in oysters injected with India ink at 12° to 21° C. (Stauber, 1950) and 2 to 5 days in oysters injected with yeast cells and vegetative *Bacillus mycoides* at 17° ± 1° C. (Tripp, 1960). The evidence indicates that migration of host leucocytes through epithelial surfaces is a normal physiological process. However, under experimental conditions, this process could be accentuated or retarded, depending on the size and number of particles phagocytosed or pinocytosed by the leucocytes and the susceptibility of particles to intracellular digestion. Lack of protein-laden leucocytes migrating through the arterial wall could be ascribed in part to the relatively early wide distribution of the labelled proteins to the terminal branches of the circulatory system and, therefore, occlusion of the major vessels, if it occurs, is probably very transient.

On the basis of the present findings and observations made by other investigators, pinocytosis as displayed by various tissue cells and certain protozoa is now well established. In the oyster, the pinocytic activity of leucocytes appears to be primarily defensive, since protein-laden leucocytes are seen to traverse the epithelium of the mantle and intestine on their way to exterior. In *P. lophurae* and *P. berghei*, pinocytosis is a means of securing nutrient (Rudzinska and Trager, 1957, 1959). However, the significance of this process is still unclear in amoebae and malignant cells. Lewis (1931) assumed that digestion occurs within the pinocytosis vacuoles of amoebae. Recent studies indicate that low molecular weight substances, *e.g.*, glucose and methionine, carried inside the amoebae by pinocytosis are probably utilized (Chapman-Andresen and Holter, 1955; Chapman-Andresen and Prescott, 1956), while the fate of high molecular weight materials, *e.g.*, proteins, is still unknown. In the free-living amoebae, such as *C. chaos,* possessing a trait such as pinocytosis probably enhances the survival of the species, especially during the period when the external environment becomes hypertonic to the amoeba's "milieu interieur." Hence, it is possible that pinocytosis in the free-living amoeba could be employed as a defense mechanism against dehydration. Parasitic amoebae, on the other hand, are bathed constantly in a medium of tissue debris, red cells, bacteria and plasma, and one may infer that these organisms enrich themselves by employing pinocytosis and phagocytosis simultaneously in securing both soluble and particulate materials in the abscess. In conclusion, pinocytosis may occur as a means of defense, of obtaining nutrients or as a cytopathological manifestation of malignant cells.

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SUMMARY

In vivo and *in vitro* studies indicated that bovine hemoglobin, diphtheria antiserum, rhodamine-labelled human gamma globulin, human albumin fraction V and *Limulus* serum proteins were pinocytosed by oyster leucocytes. The presence of the various proteins within the leucocytes was detected by visual inspection of the washed sedimented leucocytes, serological techniques and fluorescence microscopy. When the proteins were injected into the living oyster *via* the ventricular route, they were readily removed by migration of protein-laden leucocytes through epithelial surfaces to the exterior. The rate of *in vitro* uptake of rhodamine-labelled human gamma globulin by the leucocytes was a function of the ambient temperature and the concentration of the protein solution in which they were bathed.

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ACTIVE MOVEMENTS AND OTHER ASPECTS OF THE BIOLOGY OF ASTICHOPUS AND LEPTOSYNAPTA (HOLOTHUROIDEA)[1]

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The ability of certain benthic sea cucumbers to execute relatively rapid movements has not been generally recognized or given adequate treatment in comprehensive accounts of the Holothuroidea (*e.g.,* Ludwig, 1892; Cuénot, 1948; Hyman, 1955). In some bathypelagic species the performance of rapid progressive movements is regarded a normal means of locomotion. Ludwig (1892) and Hansen and Madsen (1956) have noted the remarkable swimming movements, first observed by M. Sars (1868), of *Bathyplotes natans* (=*Stichopus natans*), an aspidochirotid of the typically deep-sea family Synallactidae. Gilchrist (1920, p. 381) observed that ". . . some of the Holothurians procured in deep water off the South African coasts have the power of swimming about freely in the water by an undulatory movement of the body, ..." He further surmised that ". . . such deep-sea Holothurians do not bury themselves in the soft mud of the floor of the ocean, but flit more or less readily over its surface." More recently, Hansen and Madsen (1956, p. 55) have suggested, "Probably a power of swimming, though often awkward, may be attributed to a considerable number of Holothurians of the family Psychropotidae within the order Elasipoda and of the genera *Bathyplotes* and *Paelopatides* within the Synallactidae of the order Aspidochirota." These authors remarked that of the known bathypelagic holothurians, *Galatheathuria aspera* is probably the best adapted for active swimming, which in this species is effected through undulatory movements of the lateral brim much as in the swimming of the cuttlefish, *Sepia.* The elasipodid, *Benthodytes typica,* with a wide brim all around the body, is also well adapted for swimming.

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[1] The portion of the study dealing with *Astichopus* was supported by National Science Foundation Grant GB-888; observations on *Leptosynapta* were made by the author while employed with the California Cooperative Oceanic Fisheries Investigations. Appreciation is expressed for the aid rendered by the following persons and institutions: Charles E. Cutress, Smithsonian Institution, Washington, D. C., who supplied information on the swimming behavior of *Astichopus* and made available pertinent literature; David L. Pawson, also of the Smithsonian Institution, who made available pertinent literature and reviewed the manuscript; Alfred H. Hummel, SCUBA instructor at Ramey Air Force Base, Aguadilla, Puerto Rico, who assisted in the collection of specimens of *Astichopus;* Frank Fernández, Research Assistant, Institute of Marine Biology, who helped with the field and laboratory studies; John Shoup, Bernice P. Bishop Museum, Honolulu, Hawaii, who helped with certain phases of the behavior studies; Stan Wimberley, Geology Section, University of Puerto Rico, Mayagüez, who aided in the analysis of sediment samples; Luis M. Quiñones-Rodríguez, Department of Physics, University of Puerto Rico, Mayagüez, who supplied monochromatic lamps and filters. Credit is also due Elisabeth Deichmann, Museum of Comparative Zoology, Harvard University and Kenneth R. H. Read, Division of General Education, Boston University, for criticizing the manuscript.

Of the typically bottom-living, non-pelagic sea cucumbers, only a small number of species were known to execute relatively rapid movements. All of these species are members of the family Synaptidae in the order Apodida. As originally reported by Nutting (1919), and quoted by Fisher in Deichmann (1926), *Euapta lappa* can swim to a limited extent. Costello (1946) has described well the active, scissor-like movements of the young of *Leptosynapta albicans* (=*inhaerens*), as first reported briefly by Clark (1907, p. 63). Recently, Hoshiai (1963) has observed undulatory swimming in the young of *Labidoplax dubia*.

The quick swimming movements observed in adults of the aspidochirotid *Astichopus multifidus* and *Leptosynapta albicans* are documented for the first time in the present communication. Further, a description of some other kinds of locomotory movements performed by *Astichopus,* which were formerly unknown in the Holothuroidea, is included in this paper. Also, other aspects of the biology of *Astichopus* are investigated in relation to the species' active movements, *viz.* the nature of its habitat, the reactions elicited as a result of alterations of the immediate environment (for example mechanical disturbances, temperature, light, salinity), and its toxicity. A description of the sinusoidal swimming behavior of adult *Leptosynapta* concludes the study.

MATERIALS

Astichopus multifidus (Sluiter, 1910) is a member of the order Aspidochirotida; members of this group are characterized by possessing disk-shaped tentacles and respiratory trees. Examination of the structure of the gonad demonstrated that it occurs as two tufts, thus confirming that the species does belong to the family Stichopodidae (Deichmann, 1954). *Astichopus,* a monotypic genus in the West Indies, is easily recognized because it is very large and soft, with both dorsum and ventrum uniformly covered by hundreds of tube feet; the dorsal tube feet are papillate (Figs. 1 and 4). The dorsum of all specimens examined was some shade of brown or gray, and exhibited a variable color pattern. Two individuals had a chocolate brown dorsum with numerous small (*ca.* 1 cm. diameter) scattered white spots; the ventrum was also chocolate brown. Several specimens had a light brown dorsum, and one of these possessed in addition three large (3–5 cm. diameter), evenly spaced, chocolate brown spots. Lighter colored individuals tended to have a white ventrum. The tube feet and papillae were light in color, usually a translucent light yellow or light brown. Undisturbed, crawling *Astichopus* demonstrated a range in total length of 29–46 cm. Specimens observed by Clark (1933) were somewhat larger, at least 45 cm. in length. Aggregates of numerous minute grains and scattered C-, S-, or O-shaped calcareous particles occur in the body wall (Deichmann, 1954). Three of the specimens collected in Puerto Rico and used as material in this study have been deposited in the Smithsonian Institution, U. S. National Museum (Number E-10325).

Few specimens of *Astichopus* have been collected previously; the largest number reported were brought up in trawl hauls made on the Campeche Bank in the Gulf of Mexico. According to H. Hildebrand (quoted in Deichmann, 1954), this species is an abundant form in this region. Several specimens were also collected at Port Antonio, Jamaica, by Clark (1933). Since these earlier occurrences, no other specimens have been reported from Jamaica (Fontaine, 1953). This species has been reported from only one other locality in the tropical western Atlantic, namely at Tortugas, Florida (Deichmann, 1963); the specimens found in Puerto Rico constitute a new record for this region. Because a dense population of this comparatively rare species has been discovered in Puerto Rico, an account of the habitat is given in the next section.

Leptosynapta albicans (Selenka, 1867), a well known Californian sea cucumber, belongs to the order Apodida, a group wholly lacking tube feet, and to the family Synaptidae, whose members possess calcareous spicules in the form of anchors and anchor plates and tentacles with slender digits. The observations reported in this study were made on animals living in Monterey Bay, California.

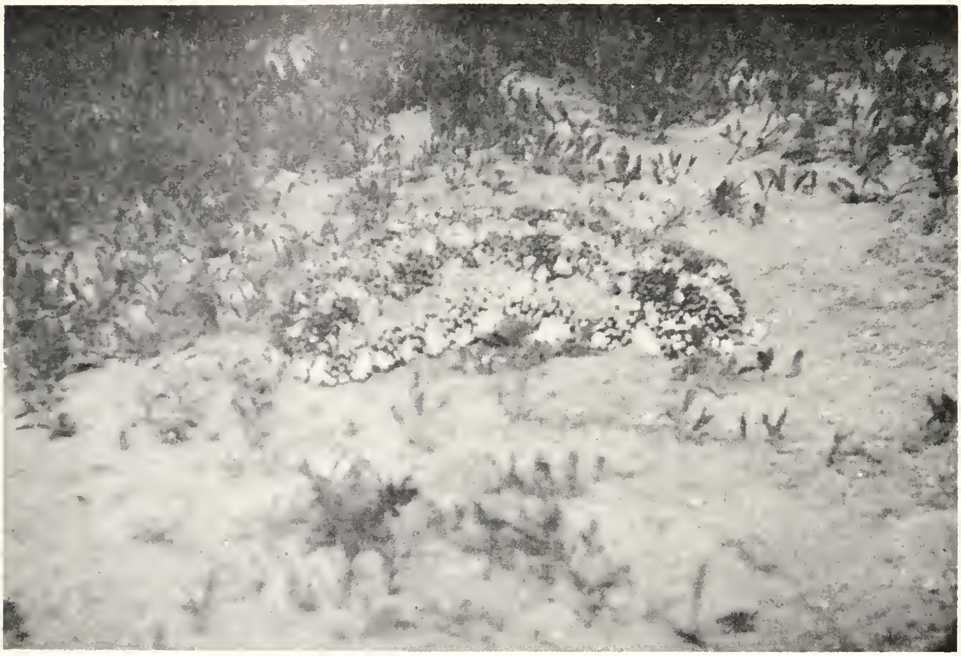

FIGURE 1. Underwater photograph of *Astichopus multifidus* at the edge of a bed of the seagrass, *Halophila baillonis,* in 15 m. of water at Crashboat Landing, Aguadilla (November 20, 1964). The length of this animal, as it is crawling in the picture, was approximately 35 cm. Forward progression is toward the right; visible are the cloacal aperture at the rear end of the animal to the left and a lateral fringe of papillae bordering the ventrum.

Limited studies were carried out on *Synaptula hydriformis* (Le Sueur, 1823), a viviparous member of the Synaptidae. This is an abundant species of the West Indian fauna, usually living associated with algae. All specimens were collected from the red alga, *Laurencia papillosa*, which grows on a sandy bottom in the shade of red mangroves (*Rhizophora mangle*) on the reef flat at Cayo Májimo, La Parguera, Puerto Rico.

Because different methods were employed in the various experiments performed, these are discussed separately under the appropriate sections to follow.

ASTICHOPUS MULTIFIDUS

Habitat

Astichopus has been found at five different localities in the coastal waters of western Puerto Rico (Fig. 2). It is most abundant on the northwestern coast, and at Crashboat Landing, midway between Pta. Borinquen and Aguadilla, several specimens have been observed on numerous occasions throughout the year. The species has been seen at depths of 20–40 m. at different times near Isabela, Camuy and Arecibo, and probably occurs in favorable localities between these areas.[2] The results of a typical collecting trip to obtain specimens of *Astichopus,* conducted at Crashboat Landing on September 29, 1964, give an approximate indication of its abundance in this locality. Three individuals were found, two at a depth of 20 m. and one at 10 m. (measured with a wrist depth gauge), by two SCUBA divers swimming abreast, searching a path 10 m. in width over a distance of about 1 km.

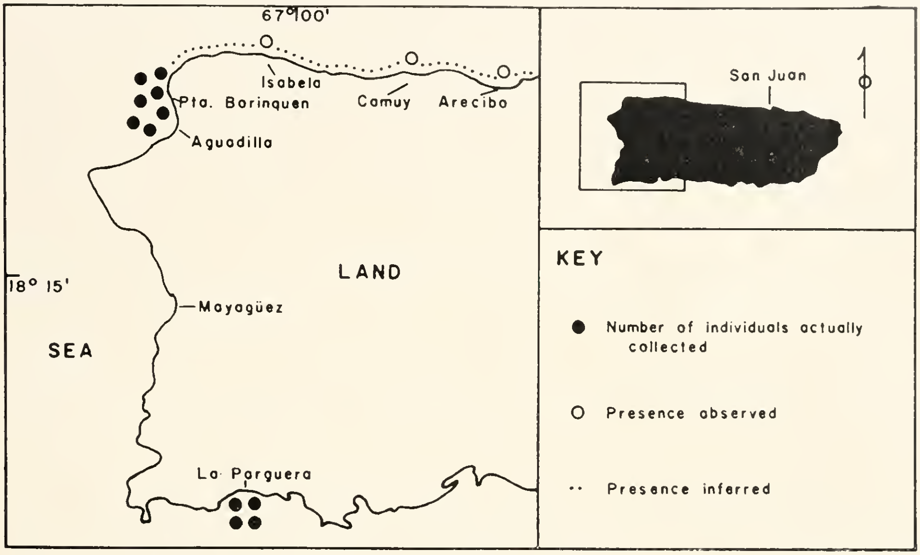

FIGURE 2. Map of western district of Puerto Rico showing the localities where *Astichopus* has been collected (solid circles) and observed (open circles) from June, 1960, to October, 1964. Its probable occurrence along the north coast is also indicated (dots). The small inset map of Puerto Rico shows the sector of the island examined.

A dense growth of the marine phanerogam, *Halophila baillonis,* was present where *Astichopus* was collected at 20 m.; the other specimen was taken from the bare sandy bottom. An underwater photograph of a cucumber on a *Halophila*-covered bottom is shown in Figure 1. The four individuals of *Astichopus* reported from La Parguera were found in the months of January, February, August and October at a depth of from 1 to 3 m., either on a bottom with a dense growth of turtle grass (*Thalassia testudinum*), or nearby on the bare sand on the leeward side between the two inshore coral reefs, Cayo Caracoles and Cayo Májimo. The present observations on the bathymetric range of *Astichopus* support Deichmann's (1963) belief that this species normally lives in deeper water, occurring at shallow depths only sporadically.

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[2] Gary E. Branham and Alfred H. Hummel informed the author of the occurrence of *Astichopus* at all localities from Pta. Borinquen east to Arecibo.

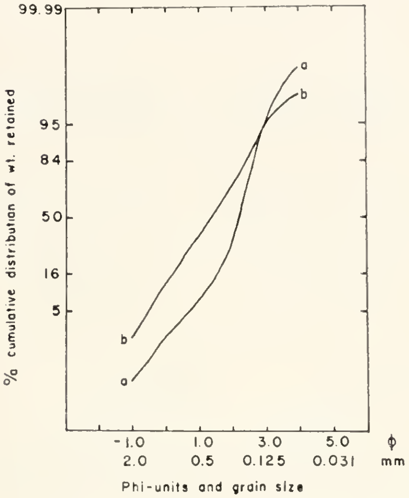
Other echinoderms observed commonly at Crashboat Landing on the sandy bottom between 30 and 45 m. of depth were the echinoids, *Astropyga magnifica* (Diadematidae) and *Meoma ventricosa* (Clypeasteridae). *Astropyga* is not reported from La Parguera and *Meoma* has been found there only infrequently.

Physically and biologically the shore line from Aguadilla to Camuy is decidedly different from that in the vicinity of La Parguera on the south coast. Kaye (1959) has described the northwestern coast, from Aguadilla to Arecibo, as largely composed of a limestone cliff, occasionally interrupted by a narrow rocky or sandy bench, which often forms a firm surface where cementation of dunes has occurred. Mangrove forests border most of the coastline around La Parguera and shallow fringing and patch reefs, composed of such prominent coral species as *Acropora palmata, Montastrea annularis* and *Porites porites* var. *furcata,* are numerous (Almy and Carrión, 1963).

Considerably stronger waves buffet the north coast of Puerto Rico than along the south shore, except during cyclonic disturbances from the southern quarter (Glynn, Almodóvar and González, 1964). The normally heavy surf along the north coast is a result of the following conditions: (a) this region is exposed directly to the high seas generated across the Atlantic Ocean, (b) the island shelf is narrow with few offshore reefs and banks, and (c) the windward shore receives large swells resulting from storms in the North Atlantic during the winter season (Kaye, 1959).

Substantial fresh-water discharge is also a prominent feature along the Atlantic seaboard of Puerto Rico. Because of the southern location of the north-south drainage divide, seven of Puerto Rico's 17 principal rivers, with an approximate drainage area of 1398 square miles or 64% of the total considered here, discharge at more or less regular intervals along the north coast (Arnow and Bogart, 1960). No permanent river system is present in the vicinity of La Parguera on the south coast.

Surface sediment samples (upper 5 cm. stratum), collected from the sites where *Astichopus* was found in greatest abundance at Crashboat Landing and Cayo Caracoles, vary considerably in grain size and composition (Fig. 3). The median diameters and degrees of sorting (as indicated by phi standard deviation; Inman, 1952) for the Aguadilla and La Parguera samples were 0.212 mm., with 1.1 phi-units and 0.392 mm., with 2.2 phi-units, respectively. The terrigenous fraction of the sample from Crashboat Landing contained mostly quartz and feldspar with about 5% heavy minerals. Calcareous bioclastic materials, constituting 97.2% of the dry weight of the sample from La Parguera, were nearly three times as great as at Aguadilla. *Halimeda* fragments were the principal constituents in the south coast sample, with the remainder composed of broken skeletons of other calcareous algae, the sessile foraminiferan, *Homotrema rubrum,* coral fragments, echinoid tests and spines and a variety of other invertebrate hard parts. The more poorly sorted sediment sample from La Parguera might be explained by the high per cent composition of plate-like *Halimeda* fragments and the location of the area on the Caribbean Sea, in the lee of the heavy swells and surf action of the Atlantic coast of Puerto Rico. Sediment analyses reported by Guillou and Glass (1957) confirm the divergent character of the substrata as revealed in the present study. Calcareous and non-calcareous materials were observed to be present in equal amounts in the beach sands from Aguadilla to Río Camuy, while inshore sediments along the southwestern coast (all of south coast as shown in Figure 2) were predominantly calcareous.



	a – Crasnboat Landing	b – Cayo Caracoles
Terrigenous residue	62.5	trace
Calcium carbonate	36.8	97.2
Organic matter	0.7	2.8

FIGURE 3. Plot on probability paper showing cumulative percent distributions of grain size in sediment samples from *Astichopus* habitats at Crashboat Landing, Aguadilla (a) and Cayo Caracoles, La Parguera (b). The per cent composition of the samples, based on dry weight, is tabulated in terms of terrigenous residue, calcium carbonate and organic matter content. Grain size distribution was determined by standard sieve analysis, calcium carbonate and organic matter contents by the difference in weights obtained after ample treatment with HCl and H_2O_2, respectively. The H_2O_2 technique employed is outlined by Stevenson and Emery (1958). Terrigenous residue, as here defined, was that portion of the sample remaining after the above treatment.

Movements

Clark (1933) observed that *Astichopus* is a very active form and remarked (p. 111) that it ". . . moved about more obviously than any other large holothurian I have ever watched." The various movements performed by *Astichopus* were studied in the field during daylight hours and in the laboratory during the day and at night. Animals maintained in captivity were kept in 200-liter DUROTEX (asbestos) troughs and 210-liter aquaria supplied with running sea water. Figure 4 illustrates some of the different movements observed, and the account of these follows.

A slow crawl is the most frequent means of progression when the animal is left undisturbed (Fig. 4a). All specimens observed in the field were crawling over the sandy bottom, ingesting the substratum by means of the circlet of large tentacles surrounding the mouth. Examination of gut contents showed that there is no apparent discrimination of particle size, and that large fragments of either living or dead plant material are generally avoided. Two cucumbers, timed while crawling under laboratory conditions and not feeding, progressed at a mean rate of 0.25 and 0.15 m./min. (Table I). Individuals observed in the field while feeding moved along at a slower rate. As indicated by the lengths measured in the two individuals included in Table I, a disturbed animal will contract to about three-quarters of its crawling length. When the body is contracted the dermal papillae (dorsal tube feet) are frequently withdrawn into the body wall. The crawl is accomplished through the forward progression of a peristaltic wave originating from the posterior end of the animal. The posterior end is first elevated two to four cm. from the substratum and then the wave moves forward, forming a two-cm.-high arch between the ventrum and the underlying surface. Of the many tube feet uniformly distributed over the ventrum, the few attached to the substratum are detached momentarily as the peristaltic wave moves forward. Three peristaltic waves passed across the body of a 40-cm individual in a mean time of 65 seconds. A second cycle is initiated by the time the first has moved over two-thirds of the body length.

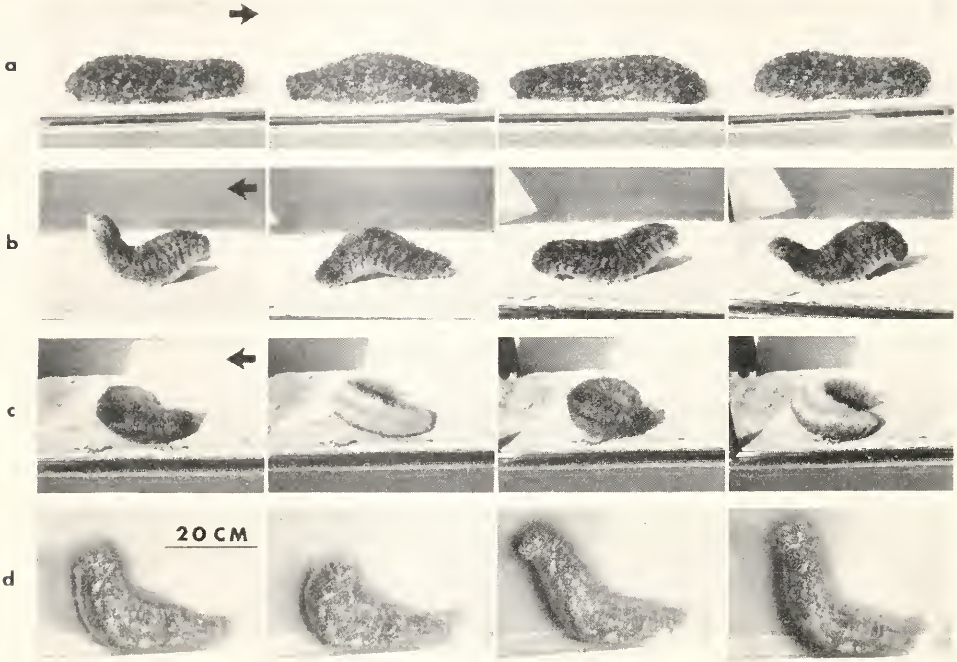


FIGURE 4. Four distinct movements performed by *Astichopus* in captivity. The four photographs in each horizontal series demonstrate (a) crawling, (b) bounding, (c) rolling and (d) an exploratory activity. Arrows in series (a) through (c) indicate the direction of forward progression, viewing the photographs in sequence from left to right. The inset scale in the first photograph in series (d) indicates approximately the sizes of the three different individuals illustrated. The actual lengths of the specimens, measured while crawling, were (a) 32 cm., (b) and (c) 30 cm., and (d) 46 cm.

Under certain conditions, to be discussed in the next section, the crawl will suddenly quicken to a walking or a bounding type of locomotion (Fig. 4b). A gradation in speed from crawling to bounding is clearly evident, but once one of the three modes of progression is executed it persists for some few minutes. Walking speeds of three-quarters to one m./min. are usual, whereas one and one-half to two m./min. are rates typical of the bounding movement (Table I). As in crawling, both walking and bounding are initiated by a peristaltic wave which moves forward along the body. However, the cycle is obviously more rapid and exaggerated at greater velocities. For example, in the bounding movement a wave passes along the entire length of the body in three to five seconds and there is no attachment of the tube feet to the bottom. As a wave terminates anteriorly, the forward end of the body is thrown upwards forcefully and at the same moment a new cycle begins at the hind end. Even at the highest speed attained, *Astichopus* always maintained contact with the bottom. Indeed, it appears that this contact is necessary for the forward thrust, since animals pushed over on their sides and still bounding remain essentially stationary. Many of the dorsal papillae contract into the skin while the animal is walking or bounding. The total distance covered by one specimen while bounding in an aquarium was about 10 m. Each time the animal reached the obstructing wall at the end of the aquarium it was turned around gently so as not to interfere with its forward progression. Animals walking or bounding tended to deviate little from a straight-line course. Only limited bounding movements have ever been observed in the field, *i.e.*, of the order of one to one and one-half m. traversed at a time.

TABLE I

Rates of progression for the crawling, walking and bounding movements of Astichopus as observed in captivity

Specimen	Type of movement (m./min.)		
	Crawling	Walking	Bounding
Light brown individual; crawling length 39 cm.; contracted length 30 cm.	0.23	0.62	1.72
	0.25	0.76	1.89
	0.26	0.88	1.98
	$\bar{X}$ 0.25	0.75	1.86
Dark brown individual; crawling length 39 cm.; contracted length 32 cm.	0.12	0.74	1.33
	0.16	0.90	1.43
	0.18	1.01	1.71
	$\bar{X}$ 0.15	0.88	1.49

A swimming-like response was observed by Cutress (personal communication) on one occasion. The animal actually left the bottom and progressed through the water in an undulatory manner. Further attempts were made to repeat the act, but they resulted only in the bounding movement already described. In the present study one individual on two different occasions was stimulated to bound in the field by subjecting the animal to a sudden change in the temperature of the water (see section on environmental effects on movements), but the bounding movement did not lead to swimming.

Rolling movements also occur frequently, and as shown in Figure 4c the body flexes into a U during this activity. A complete roll is carried out in unison along the whole length of the animal. About one-half of the dorsal papillae are contracted during this movement. One individual made several complete rolls in a mean time of five seconds. Rolling may last as long as five minutes, with the animal moving little from its original position. It became evident that all of the movements thus far described were less easily evoked in individuals recently stimulated and in some animals activity decreased in frequency and intensity with time maintained in captivity.

An exploratory or searching activity was observed on only one occasion (Fig. 4d), and this occurred in one of three individuals in the same aquarium immediately after transportation to the laboratory from the field. About one-third of the anterior portion of the body was elevated, and this swung frequently from side to side, describing an arc of approximately 40°. A full swing to the left is shown in the second photograph in Figure 4d. The exploratory movements lasted for about 10 minutes and culminated in the erect posture illustrated in photographs three and four of Figure 4d. With over one-half of the body in an upright position the animal bowed forward several times, forming an angle of 45° from the vertical.

The five longitudinal retractor muscles, which must play an important role in all of the various movements, are very well developed in *Astichopus* as 1–2-cm.-broad bands running the length of the body. Comparable muscles in a closely related, sluggish form, *Isotichopus* (= *Stichopus*) *badionotus,* are thin and narrow. The body wall is tough and thick in both species, but soft and pliable in *Astichopus* and rigid in *Isostichopus.* Excessive muscular development has also been reported in the swimming sipunculid, *Sipunculus natans* (Fisher, 1954), a member of a typically benthic group. This species has developed strong longitudinal muscle bands and enormous wing-muscles.

Environmental effects on movements

In order to learn how the various movements just described may be used to advantage by *Astichopus,* several animals were stimulated in the field and in the laboratory to produce disturbing circumstances likely to be encountered by the species in its natural surroundings. Such conditions studied were various bodily disturbances, alterations in the temperature of the water, reduction in salinity, effects of oxygen-deficient water and behavior in numerous divergent light regimes. In all instances parallel control procedures demonstrated that the movements evoked were due to the particular conditions under investigation.

Procedure involving mechanical stimulation was as follows. Two individuals were buried completely beneath the bottom sediments and elevated quickly (in 30 seconds) toward the surface, over an ambient pressure gradient of one atmosphere. All of the animals responded by contracting initially, then after a minute or two began to crawl. Gentle handling of specimens normally produces the same reaction. All of several individuals, turned over on their dorsa and sides, righted themselves immediately, in 3–5 seconds, by the rolling movement. A half- or quarter-roll only was performed to regain normal posture. Since current action on the bottom is relatively strong at Crashboat Landing, often swaying the animals from side to side down to a depth of 30 m., this quick, righting response is well suited to help maintain proper orientation.

Sudden changes in sea water temperature, of the order of 3–4° C., elicited the walking, bounding and rolling movements more effectively than any other environmental alterations investigated. To test the effects of temperature, animals were moved rapidly from one aquarium, at a lower or higher temperature, to another. In addition, some individuals were maintained in a slightly cooled or heated state in captivity, and then transferred rapidly to the field. The laboratory sea water temperatures ranged from 27.5° to 29.3° C. over the duration of the experiments. Coastal sea water temperatures at this time of the year (October) were in the range of 27–29° C. Different individuals of *Astichopus* were subjected to the following typical changes in temperature: from 29.1° C. to 32.4° C., and from 27.7° C. to 23.3° C. Essentially the same movements were performed when the animals were subjected to a temperature which was greater or less than the initial temperature. Immediate bounding was the most frequent response, lasting for one to three minutes. A rolling movement was less common, while walking was observed on relatively few occasions. Once a particular type of movement began it usually persisted until the animal slowed down to a crawl.

The behavior of *Astichopus* under different conditions of lighting was investigated with the following light sources: natural daylight, house light (tungsten filament lamp), red light (infrared lamp), yellow light (sodium lamp), filtered green light, blue light (mercury lamp) and violet light (ultraviolet lamp). The light beam was directed so as to enter a 210-liter aquarium on one side from above; cucumbers were placed lengthwise along the edge of the lighted portion and the shadow of the dark side of the aquarium, which was covered with black cloth. Observations were made in a darkroom where the light intensities employed were equal to or less than 10 foot-candles. Light intensities up to 75 foot-candles were measured with a Weston photoelectric cell (Model 703, sight meter); an estimation of greater illumination was obtained by the interposition of green filters over the photocell and by calibrating the Weston meter against a General Electric Mascot exposure meter (Type PR-35).

Individuals showed a marked attraction to natural and artificial light of low intensity ($\leq$5 foot-candles), by immediately crawling toward the source. No consistent phototactic attraction or repulsion could be discerned at higher light intensities of 10, 20, 30, 50 and even 11,000 foot-candles (zenith sun at noontime in November). Usually, however, activity did increase at higher illuminations. In contrast to the usual photo-negative response of holothurians (Crozier, 1915) *Astichopus* crawled with equal frequency toward and away from the illuminated end of the aquarium. The phototactic behavior of *Astichopus* seemingly parallels that of *Isostichopus,* a form not apparently irritated by a strong source of illumination.

Monochromatic light was adjusted so that a maximum intensity of 10 foot-candles entered through one side of the aquarium. *Astichopus* demonstrated a strong, positive attraction to red, green, blue and violet light; individuals moved toward the light immediately by crawling and lingered in the region of greatest illumination. Only a modest attraction to yellow light was observed.

Astichopus did not demonstrate any clear tendencies in geotactic or thigmotactic behavior. Animals climbed readily up and down the vertical sides of containers and surfaces inclined at various angles to the horizontal. Occasionally individuals in the field were found alongside submerged pipes and pilings. Movements of confined laboratory animals indicate, however, that the association with solid objects may occur simply by chance wandering.

Effects resulting from a reduction in salinity were observed in the laboratory in individuals transferred from the sea water in which they were maintained to tanks containing sea water diluted with tap water. The normal, mean, surface salinity on the inshore reefs of the south coast for November, when these particular studies were made, was around 34.50‰ (unpublished data). Experimental dilutions were 31.00‰ and 17.25‰. Animals suddenly subjected to these conditions usually contracted, but occasionally executed a bounding motion. An equal intensity of reaction, in terms of speed and duration, was noted in both dilutions.

Individuals of *Astichopus* were also transferred to oxygen-deficient sea water—obtained directly over anaerobic mud on the floor of a mangrove canal. Of the rapid movements a bounding response was elicited most frequently under these conditions; limited rolling was also observed.

The possibility that the association of *Astichopus* with members of its own species or with other organisms may elicit active movements was also investigated. Individuals together in the same container behaved independently, *i.e.*, they crawled about as if alone and tended to avoid contact with other cucumbers. Animals introduced into an aerated aquarium in which *Astichopus* was previously living showed no signs of excitation. The water in this case was recirculated through a filter instead of being replaced by fresh, running water. Simulated biting, by firm pinching of different regions of the body with the bare hand, was observed both in the field and in the laboratory. This evoked a typical contraction of the body with subsequent crawling after 1–2 minutes. Introduction of a fresh slime preparation from the skin of *Lactophrys bicaudalis*, a trunk-fish which regularly preys on the small holothurian, *Microthele* (= *Holothuria*) *parvula*, in the immediate vicinity of *Astichopus* also failed to produce any active movements.

Two observations indicated that *Astichopus* might possibly possess a toxic substance that adversely affects other organisms nearby. *Petrochirus diogenes*, a large scavenging and possibly predatory hermit crab (Randall, 1964) found in association with *Astichopus*, tended to avoid the cucumber in captivity. In addition, several fishes in the same aquarium with *Astichopus* died after one cucumber eviscerated.

Toxicity

Yamanouchi (1955) has clearly demonstrated that numerous species of holothurians are toxic; of 27 species investigated, 24 contained a venomous substance. Nigrelli and Jakowska (1960) reported that poisonous species are known in four of the five orders comprising the class; members of the deep-sea order Elasipodida have not been examined in this connection. The total number of species of cucumbers known to be toxic to fishes is 30—24 of these live in the Pacific Ocean, three in the Mediterranean and four in the West Indies (Bahamas). One of the latter species occurs in the Mediterranean and in the West Indies. The toxic principle in the common Bahamian species, *Actinopyga agassizi,* has been identified as a saponin, a chemical structure previously unknown in the animal kingdom (Nigrelli *et al.,* 1955).[3]

Investigations were made of the possible adverse effects on other marine animals of (a) the water in which *Astichopus* had recently performed active movements, (b) its coelomic fluids, and (c) alcohol extracts of the body wall. Freshly killed cucumbers were used in all instances. The potency of *Astichopus* was assayed by observing the effects produced in seven species representative of six different animal phyla: Coelenterata, Madreporaria—*Porites porites* var. *furcata* Lamarck, 1816; Annelida, Polychaeta—*Hesione proctochona* Schmarda, 1861; Mollusca, Pelecypoda—*Lima scabra* Born, 1778; Arthropoda, Crustacea—*Mithrax* (*Mithraculus*) *sculptus* (Lamarck, 1818); Echinodermata, Ophiuroidea—*Ophiothrix angulata* Say, 1825; Chordata, Tunicata—*Ecteinascidia turbinata* Herdman, 1880; Chordata, Pisces—*Jenkinsia lamprotaenia* (Gosse, 1851). All of the invertebrate species were held in wide-mouth bowls with one liter of aerated sea water at a temperature of 28–29° C.; *Jenkinsia* was held in a circular bottle of 10 liters capacity. All invertebrates except *Ecteinascidia* were collected on the same day of an experiment from the south shore of Magueyes Island, in association with the fringing *Porites porites* var. *furcata* reef. *Ecteinascidia* was collected from mangrove roots bordering the canal which separates Magueyes Island from the mainland; *Jenkinsia* was netted near the shoreline of the same canal. Parallel control material, with at least the same number of individuals observed as in experimental, always accompanied each experiment.

Two 10-ml. samples of sea water, taken from separate 5-liter containers in which one *Astichopus* had rolled and another bounded, did not produce any visible effects on the test animals over a 24-hour period.

An unsuccessful attempt was made to stimulate evisceration in three different individuals by firmly squeezing and pinching various areas of the body. Further, evisceration did not occur under the diverse experimental conditions to which *Astichopus* was subjected. Only two individuals eviscerated over a two-month period and the cause was not readily apparent. Much of the gut and the respiratory trees were ejected; Cuvieran organs are unknown in all Stichopodidae. For these reasons, it does not appear likely that evisceration is a normal defensive response. The body fluids tested for toxicity, then, were obtained by dissection from the coelomic cavity. Undiluted coelomic fluid, equal to a final concentration of 1000 ppm. (parts per million), was added to the water in which the test species were confined, and their reactions observed continuously for the first two hours after introduction and then at 4 hours and 8 hours. At the termination of the experiment animals were transferred to running sea water for a duration of 12 hours in order to observe recovery. The various responses noted are summarized in Table II.

Polyps of the scleractinian coral *Porites* contracted slightly in the first one-half hour and remained in this state for 8 hours. In 4–8 hours a mild lethargic response was observed in *Hesione, Mithrax* and *Ophiothrix;* they all became less irritable to mild mechanical stimulation. *Lima* reacted immediately by violently closing its valves; after about one hour they began to gape. By two hours the water became noticeably reddened and contained numerous small fragments of mantle tissues voided by the animals. *Ecteinascidia* responded by slowly contracting until at 8 hours all individuals were dead; cessation of heart beat was used as a criterion of death. *Jenkinsia*, the small clupeid, perished quickly; two fish were dead at the end of the first hour and all five had succumbed in two hours. The five control individuals of *Jenkinsia* lived beyond the 8 hours of the experiment. Recovery occurred in four of the five surviving species; *Ophiothrix* died.

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[3] Studies on the chemical nature of the toxic agent are presently being carried out by the author in collaboration with Horace Graham, Department of Biology, University of Puerto Rico, Mayagüez.

TABLE II

Observations of the effects on some marine animals of the body fluids of Astichopus at a concentration of 1000 parts per million

Organism	Number observed	Reactions at indicated time intervals					Recovery (12 hrs.)**
		½ hr.	1 hr.	2 hrs.	4 hrs.	8 hrs.	
Coelenterata *Porites porites* var. *furcata*	3 terminal branches, over 100 polyps in each	polyps slightly contracted	same	same	same	same	yes
Annelida *Hesione proctochona*	3	normal*	normal	normal	loss of quick wriggling response	same	yes
Mollusca *Lima scabra*	5	immediate and complete closure of valves	valves open in 2 individuals	all open; small bits of mantle tissue ejected	same	same	yes
Arthropoda *Mithrax (Mithraculus) sculptus*	4	normal	normal	normal	loss of quick defensive reaction of chelae	same	yes
Echinodermata *Ophiothrix angulata*	5	normal	normal	normal	normal	sluggish	no
Chordata-Tunicata *Ecteinascidia turbinata*	1 colony of 20 individuals	normal	siphons slightly contracted	same	siphons and body wall contracted	all dead; siphons and body wall greatly contracted; no heart beat	—
Chordata-Vertebrata *Jenkinsia lamprotaenia*	5	normal	2 dead	all dead	—	—	—

* A normal reaction indicates that no difference could be discerned between the test animals and controls. Same applies to Tables III and IV.

** Twelve hours in running sea water were allowed for recovery after the termination of the experiment. Same applies in Tables III and IV.

Yamanouchi (1955) and Nigrelli and Jakowska (1960) have extracted with hot ethanol an active, toxic principle from the body wall of various holothurians. Alcohol extracts obtained from *Astichopus* were prepared as follows. A one-inch square of the body wall was macerated in a mortar with 30–40 mesh quartz sand. The mash and juices were then treated with 50 ml. of 95% hot ethanol at 50–60° C. for 10 minutes. The extracts at concentrations of 1000 and 5000 ppm. were tested after cooling to room temperature. The results are presented in Tables III and IV.

The reactions of the test animals to alcohol extracts were very similar to those resulting from exposure to body fluids. Usually, however, a more severe reaction was evoked by the extract when equal to or at a greater concentration than the body fluids. The slightly contracted polyps of *Porites* after one hour were completely withdrawn in two hours. Lethargy again occurred in *Hesione, Mithrax* and *Ophiothrix*. Additional effects observed after 8 hours were inflation of the body and eversion of the proboscis in *Hesione* and extensive autotomy of the arms in *Ophiothrix*. Violent swimming movements were executed by *Lima* in the first few minutes, followed by closure of the valves. Individuals began to open after 1–2 hours; the pallial tentacles were noticeably shortened and constricted; reddened water, apparently due to the leakage of blood through ruptured tissues, appeared after two hours. All specimens of *Lima* succumbed during the experiment. *Ecteinascidia* responded by severe muscular contraction and cessation of heart beat at a concentration of 5000 ppm. Rapid, erratic swimming commenced in *Jenkinsia* immediately, resulting in the death of all specimens within 10 minutes. The gills were reddened in fish subjected to a concentration of 5000 ppm., indicating hemorrhage of the capillaries. A similar cause of death was observed in

TABLE III

Observations of the effects on some marine animals of an alcohol extract of the body wall of Astichopus at a concentration of 1000 parts per million

Organism	Number observed	Reactions at indicated time intervals					Recovery (12 hrs.)
		½ hr.	1 hr.	2 hrs.	4 hrs.	8 hrs.	
Coelenterata *Porites porites* var. *furcata*	3 terminal branches, over 100 polyps in each	normal	polyps slightly contracted	polyps fully contracted	same	same	no
Annelida *Hesione proctochona*	5	normal	normal	normal	loss of quick wriggling response	body inflated; proboscis everted	yes
Mollusca *Lima scabra*	5	active swimming followed by complete closure of valves	valves partially open; tentacles constricted	small bits of mantle tissue ejected	moribund	all dead	—
Arthropoda *Mithrax (Mithraculus) sculptus*	5	normal	normal	normal	loss of quick defensive reaction of chelae	same	yes
Echinodermata *Ophiothrix angulata*	5	normal	normal	sluggish	same	autotomy of arms	no
Chordata-Tunicata *Ecteinascidia turbinata*	1 colony of 20 individuals	normal	siphons and body wall slightly contracted	same	same	siphons and body wall greatly contracted	no
Chordata-Vertebrata *Jenkinsia lamprotaenia*	5	erratic swimming followed by death in 10 minutes	—	—	—	—	—

the killifish, *Cyprinodon baconi,* after exposure to an aqueous preparation of the toxic agent of *Actinopyga agassizi* (Nigrelli, 1952). Five species survived and two recovered from exposure to the extract at 1000 ppm., whereas of the four surviving species at 5000 ppm. only the majid crab, *Mithrax,* recovered.

TABLE IV

Observations of the effects on some marine animals of an alcohol extract of the body wall of Astichopus at a concentration of 5000 parts per million

Organism	Number observed	Reactions at indicated time intervals					Recovery (12 hrs.)
		½ hr.	1 hr.	2 hrs.	4 hrs.	8 hrs.	
Coelenterata *Porites porites* var. *furcata*	3 terminal branches, over 100 polyps in each	normal	polyps slightly contracted	polyps fully contracted	same	same	no
Annelida *Hesione proctochona*	5	normal	normal	less active	sluggish; loss of quick wriggling response	body inflated; proboscis everted	no
Mollusca *Lima scabra*	5	active swimming followed by complete closure of valves	valves tightly closed	2 dead; 3 moribund; valves partially open; small bits of mantle tissue ejected	4 dead; 1 moribund	all dead	—
Arthropoda *Mithrax* (*Mithraculus*) *sculptus*	3	normal	normal	normal	loss of quick defensive reaction of chelae	same	yes
Echinodermata *Ophiothrix angulata*	5	normal	normal	sluggish	same	autotomy of arms	no
Chordata-Tunicata *Ecteinascidia turbinata*	1 colony of 20 individuals	normal	siphons and body wall slightly contracted	all dead; siphons and body wall greatly contracted; no heart beat	—	—	—
Chordata-Vertebrata *Jenkinsia lamprotaenia*	5	erratic swimming followed by death in 10 minutes; gills reddened	—	—	—	—	—

LEPTOSYNAPTA ALBICANS

On three different occasions over the period January-May, 1957, four adult individuals of *Leptosynapta albicans* were observed swimming at night light stations in Monterey Bay, California. During that time of year, night light stations were occupied at approximately weekly intervals. All observations were made on dark nights, between 9:00 and 11:00 PM, from a float secured to the Monterey Municipal Pier. The sea bottom is sandy below the float and about 7 m. deep (measured from mean sea level).[1] The direction of swimming was along the outer border of visibility illuminated by a 200-watt lamp suspended in the water—about three m. away from the observer.

The swimming animals described a sinusoidal path as they moved through the water near the surface (Fig. 5). It was not possible to determine whether the progressive waves of activity occurred in the lateral or dorso-ventral plane. While swimming the cucumbers were extended in length to about 20 cm., but when captured quickly contracted to about 5 cm. It is estimated that a complete wave passed from the head to the tail end in about two seconds, and that the animals moved through the water at a rate of one meter per minute. Individuals appeared to move in the direction of the tentacular crown, although this requires verification. Participation of the tentacles in swimming appeared insignificant. Hoshiai (1963) observed that *Labidoplax* turns its anal end in the direction of movement. Like the swimming young of *Leptosynapta* (Costello, 1946) and *Labidoplax,* captured adult *Leptosynapta* did not void an appreciable amount of fecal material. Three specimens dissected open showed no signs of recent gonadal activity.

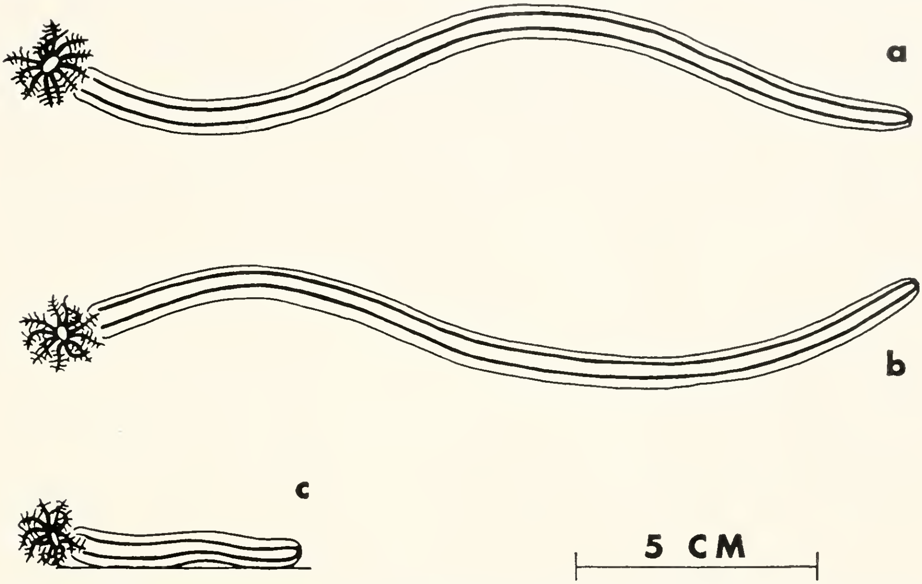

FIGURE 5. Diagrams of the undulatory swimming movements performed by *Leptosynapta* in the field (a and b) and the more frequent appearance of the animal when crawling (c).

All netted specimens were transported to the laboratory and maintained in an aquarium with running sea water. To induce swimming the same animals were treated in the following ways: (a) maintained in natural and total darkness, (b) subjected to sudden changes in light intensity (the most severe of these involved transfer from a dark room to direct sunlight), (c) dropped through a four-foot column of water, (d) subjected to a sudden increase or decrease of water temperature (14 ± 4° C.), (e) subjected to electric shocks (three graded series of voltages at 1, 10 and 100, each delivered at frequencies of 1, 10 and 100 per second), (f) subjected to a sudden increase in concentration of acid (HCl) or base (NaOH); two to three drops of 18 N HCl and a saturated solution of NaOH (*ca.* 30 N) were introduced separately into one end of a small pan containing the cucumber. An attempt was always made to keep deleterious stimuli at a sublethal level. None of the above treatments elicited a swimming response. In most cases the cucumbers tended only to avoid the stimuli by contracting the body and tentacles.

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[1] This area has not been re-examined since the construction of a nearby breakwater and small craft shelter.

Since swimming movements have been reported for three species of apodid holothurians, *Synaptula hydriformis*, a readily available species belonging to the same group, was also investigated. Stimuli identical to those enumerated above were administered to several individuals of *Synaptula*. In addition, a fresh slime preparation from the skin of the trunk-fish, *Lactophrys bicaudalis*, was introduced into an aquarium containing several individuals of *Synaptula*. The sea water temperature during the experiment was 28 ± 4° C. As in *Leptosynapta*, none of these treatments prompted a swimming or otherwise active response. The reactions were similar to those observed by Olmsted (1917), involving muscular contraction of various parts of the body.

DISCUSSION

It is of interest to contrast the habitat of *Astichopus* in Puerto Rico with that portion of the Campeche Bank in the Gulf of Mexico where the species also occurs in abundance. Numerous specimens are commonly encountered in the shrimp grounds on the Campeche Bank, from a few fathoms down to a depth of 20 m. The location of the shrimp grounds in this region (Hedgpeth, 1954, p. 206), when compared with Lynch's (1954, p. 79) map of the sedimentary provinces, shows that the bottom deposits are essentially muddy. The usually muddy and estuarine conditions where shrimps are found along the Gulf coastal states, including a high organic matter content, contrast notably with the environment of the north coast of Puerto Rico. However, there is evidence that the faunal composition of the more southern shrimping grounds is decidedly different from that in the northern Gulf (Hedgpeth, 1953, 1954), perhaps reflecting significantly different physical and chemical properties of the environment as well. Kornicker *et al.* (1959), who compiled a list of the biota of Alacrán Reef, located near the center of the Campeche Bank and away from muddy deposits, did not report the presence of *Astichopus* or the seagrass, *Halophila*.

The relationship between grain size and the distribution of benthic feeding types suggests a further complication in an attempt to delineate the set of environmental factors favorable to the species. Sanders' (1958) findings, that clay is a good sediment correlate in the distribution of deposit-feeding organisms, would seem to apply where *Astichopus* occurs in very fine sediments on the Campeche Bank. However, McNulty *et al.* (1962), in agreement with the present findings, observed that deposit feeders were most abundant in sediments with a median grain size of about 0.25 mm. On the other hand, individuals of *Astichopus* with a dry body weight (excluding ingested materials) of the order of 100 gm. do not lie on the curve relating body size to grain size proposed by McNulty and co-workers for deposit feeders. According to their findings, which show a linear relationship between grain size and the cube root of dry tissue weight, one would expect *Astichopus* to occur in sediments with a median grain diameter in excess of 0.8 mm. The delicate and highly dendritic structure of the tentacles and the way these are employed in ingesting fine sediments indicate that feeding may be more efficiently executed on fine-grained substrata.

Deichmann (personal communication) has observed that many species of cucumbers go shorewards during the breeding time. However, the four individuals of *Astichopus* found in shallow water in La Parguera did not have the gonads in an active condition. Possible breeding in the late autumn or early winter is suggested by the occurrence of a 5-mm. *Astichopus* in shallow water in the Bahamas in May (found by C. Fernández Mosher). La Parguera specimens were collected in the winter, summer and autumn seasons. The present scanty records indicate that the occurrence of *Astichopus* at shallow depths is not associated with breeding.

The walking, bounding, rolling and erect exploratory movements of *Astichopus* represent newly-described activities for the benthic Holothuroidea. Even the relatively slow crawl of this highly active species is rapid compared with other forms. The mode of crawling in *Parastichopus parvimensis* (Parker, 1921) resembles very closely that in *Astichopus,* but individuals of nearly equal size progressed at a rate of only 1 m./15 min., or 0.07 m./min., equivalent to one-fourth to one-half the speed in *Astichopus.* Peristaltic waves pass along the bodies of the two species at the same rate, *viz.* at mean times of 63 seconds in *P. parvimensis* and 65 seconds in *Astichopus.* In *Parastichopus* one peristaltic wave at a time passes over the body; in *Astichopus* a second cycle begins before the first has ended.

The swimming movements of the species *Bathyplotes natans* were described by M. Sars (Hansen and Madsen, 1956) as similar to those of swimming leeches. The snake-like bends of the body occurred in the dorso-ventral plane, not side-wise. Assuming that the progressive peristaltic waves were initiated from behind in *Bathyplotes* (in leeches the waves pass back along the body from the head) it is possible that the swimming observed in *Astichopus* by Cutress is very similar to that reported by Sars.

A speed of nearly 2 m./min. attained by *Astichopus* when bounding, approaches the greatest velocities observed among echinoderms, *viz.* comatulid crinoids, 5 m./min.; *Centrostephanus longispinus* (echinoid), 2.1 m./min.; *Crossaster papposus* (asteroid), 2 m./min.; ophiuroids, 1.8 m./min. (Hyman, 1955). Prosobranch gastropods, which, like sea cucumbers, progress by means of peristaltic waves, are considerably slower; one rapid crawler, *Thais rustica,* attained a maximum velocity of 0.3 m./min. (Coomans, 1961).

The fast movements performed by *Astichopus* may actually facilitate a more rapid adjustment to certain adverse conditions encountered naturally. As pointed out earlier, a rolling movement is used by the cucumber for stabilization in strong currents. The method by which a bounding movement could help *Astichopus* avoid conditions of high temperature stress is suggested by the following possible circumstances. Reactions to light and gravity indicate that *Astichopus* is capable of moving readily into shallower or deeper water. Also, this species is very sensitive to a sudden change in the sea water temperature; such a change will immediately evoke a bounding response. Protected, shallow bodies of water heat up considerably during mid-day low water phases of the tide in the summer. For example, on one occasion in La Parguera a temperature difference of 8° C. was observed between shoal water on the lee side of a reef (36° C.) and the surf zone to windward (28 ° C.). A cucumber wandering into such heated shallows could possibly escape the high temperature if the bounding movements were executed and properly directed. Abrupt changes in temperature are probably not so frequently experienced where *Astichopus* occurs at greater depths. Bathythermograph curves, obtained from stations located slightly less than one mile west of Pta. Borinquen, show that a distinct thermocline occurs as deep as 90–120 m., with a temperature gradient of 3° C. over about 7 m. (Gilbert Bane, personal communication).

Bounding sometimes resulted under experimental conditions when animals were suddenly subjected to reduced salinities and oxygen-deficient water. Although ample data are not available on the patterns of salinity and oxygen distributions around the river mouths on the north coast, aerial views show turbid river discharge extending seaward 2–3 miles and as streaks up to 5 miles along the coast. The effects of such conditions on populations of *Astichopus* will be the subject of a future investigation.

Aside from an exploratory type of behavior, observed on only one occasion in captivity (in the presence of two other animals), active movements are not elicited in *Astichopus* through association with members of its own species. Exposure to the juices of a possible predator (*Lactophrys bicaudalis*) also failed to arouse the cucumber. Sund (1958) likewise could not demonstrate that certain supposed predatory starfishes were responsible for quick swimming movements performed by the actinian, *Stomphia coccinea*.

Water in which *Astichopus* had performed movements did not have a toxic effect on other animals, thus showing that increased activity does not cause the release of a toxic substance. The coelomic fluid caused death in a species of brittle star, tunicate and fish at a concentration of 1000 ppm. However, the likelihood that body fluids are released naturally does not seem great, since *Astichopus* is not inclined to eviscerate even under extreme irritation. Alcohol extracts of the body wall proved to be more toxic than the coelomic fluids; five of the seven species tested perished at a concentration of 1000 ppm. Yamanouchi (1955) concluded that the poisons contained in holothurians apparently have little ecological significance. Though the toxic principle is confined largely to the tissues of the cucumber, a possible role of influencing the activities of other animals through diffusion of trace amounts into the surrounding water, for example in averting potential predators, cannot be dismissed.

Costello's (1946) description of nocturnal swimming by the young of *Leptosynapta* has dispelled the common notion that this species passes its entire post-planktonic life completely buried in soft bottoms (Hyman, 1955, p. 209). Moreover, the undulatory swimming movements performed by adult *Leptosynapta* show that Costello's suggestion is not true, namely that (1946, p. 95) ". . . *Leptosynapta* swims only during a limited period of its young adulthood." The scissor-like body flexures in the swimming young are entirely different from the sinusoidal undulations which move across the entire length of the body in adults. In addition, the movements of the young were described by Costello as more or less aimless, with a velocity of 5–6 cm./min., whereas in adults swimming was directed and occurred at a velocity of about 1 m./min.

Specimens of *Labidoplax dubia,* like adult *Leptosynapta* species, also swim in an undulatory manner (Hoshiai, 1963). Curiously, though, this species holds its anal end highest and toward the direction in which it is moving. *Labidoplax* swims from early June to late July during any lunar phase, but only after darkness; swimming begins one hour after sunset and ends one hour before sunrise. By inducing several individuals to swim in the dark during the day, Hoshiai clearly substantiated Costello's idea that swimming in synaptids is a dark-conditioned phenomenon. Darkness and a variety of other experimental procedures did not elicit swimming in adult *Leptosynapta.* It does not seem likely that the swimming behavior is related to spawning, since the gonads showed no signs of being in a ripe condition or recently spent. Furthermore, Runnström observed that *Leptosynapta albicans* erects itself only part way out of the burrow when spawning (Hyman, 1955, p. 176). In the light of present knowledge no definite statement can be made about the stimulus that evokes swimming in synaptids or the possible benefit received.

SUMMARY

1. Aspects of the biology of the aspidochirotid, *Astichopus multifidus* and the apodid, *Leptosynapta albicans,* studied in Puerto Rico and California, respectively, were investigated in relation to the active movements performed by these species.

2. *Astichopus* is present in greatest abundance between 10 and 40 m. of depth on the northwestern coast of Puerto Rico. It often occurs in or near beds of the marine phanerogam, *Halophila baillonis.* Sandy beaches, cemented dunes, and beach rock, exposed to the heavy seas of the Atlantic Ocean, make up the shoreline of this region. Numerous large rivers loaded with terrigenous materials discharge on the north shore. The sediment on which *Astichopus* lives is comparatively fine-grained (median diameter = 0.212 mm.) and well sorted ($\sigma_\varphi = 1.1$); the terrigenous component is high (62.5%), calcareous bioclastic materials occur in substantial amounts (36.8%), and the organic matter content is low (0.7%). A smaller number of *Astichopus* has been collected from shallow water (1–3 m.) in the winter, summer and autumn at La Parguera on the south coast.

3. In addition to a comparatively fast crawl, forward progression in *Astichopus* is executed by rapid walking and bounding movements, which in the latter case may approach a rate of 2 m./min. Rolling and exploratory movements are also performed by *Astichopus.*

4. Mechanical stimulation usually causes *Astichopus* to contract for 1–2 minutes. Walking, bounding and rolling movements are elicited by sudden changes in the temperature of the water, of the order of 3–4° C. A positive phototactic response occurs at low light intensities (≤ 5 foot-candles); phototaxis increases at higher light intensities, but no definite negative response is apparent. A strong positive attraction to red, green, blue and violet light is evident at a low intensity of 10 foot-candles. No clear tendencies were noted in geotactic or thigmotactic behavior. Bounding movements sometimes occurred when cucumbers were suddenly subjected to diluted sea water and oxygen-deficient water. Active movements were not evoked through the association of *Astichopus* with members of its own species or in the presence of other animals.

5. Coelomic fluids and alcohol extracts of the body wall of *Astichopus* are toxic to a variety of marine animals at concentrations of 1000–5000 ppm. It does not seem likely that a poison is released by the animal naturally, since the water in which active movements are performed is non-toxic and evisceration occurs only rarely.

6. Sinusoidal swimming movements were observed in adult *Leptosynapta* on three different occasions near the surface at night. Specimens subjected to a variety of experimental conditions in captivity failed to elicit the swimming response. *Synaptula hydriformis*, a related West Indian species, did not swim either when subjected to diverse stimuli as with *Leptosynapta* or when exposed to the juices of a presumed predatory trunk-fish.

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THE BIOLOGY OF ASCIDIA NIGRA (SAVIGNY). III. THE ANNUAL PATTERN OF COLONIZATION[1]

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In the first paper in this series (Goodbody, 1962) an account was given of the development and survival of a single population of ascidians at Port Royal, Jamaica. In October, 1959, a second experiment was initiated with the object of comparing populations which settled and commenced life at different seasons of the year. The present paper deals solely with aspects of colonization and supports the view previously expressed that *Ascidia nigra* is a primary colonizer in the sessile community and only succeeds with difficulty in developing in the later stages of community succession. A subsequent paper will deal with survival in the same population.

METHODS

The methods used for obtaining populations were exactly the same as described previously (Goodbody, 1962). Panels of Tufnol with an available settlement area of 216 sq. in. on each side were suspended from rafts at the same locality and in the same manner as before; natural sessile communities, which included populations of *Ascidia nigra*, were allowed to develop on the panels, which were inspected at monthly intervals. Altogether six separate populations were set up in this way and were designated A, B, C, D, E and F. Four panels were used for each population, two sited at four feet and two at eight feet below the sea surface. The populations were set up so that they commenced life at intervals of two months over a period of a year; this was accomplished by immersing the sets of panels at successive intervals as follows:

Population	Date of panel immersion
A	1st October 1959
B	1st December 1959
C	1st February 1960
D	1st April 1960
E	1st June 1960
F	1st August 1960

After immersion every panel was examined and photographed at monthly intervals and a permanent record thus obtained of the fate of every individual ascidian. Records of settlement continued until March, 1962, and of survival until October, 1962. Population C suffered damage and handling loss in the winter of 1960/61 and observations on it were abandoned after January, 1961.

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[1] Supported in part by a grant from the Nuffield Foundation.

THE PATTERN OF COLONIZATION

Table I shows the number of new colonizers recorded in each population in each month throughout its history. It should be noted, however, that no counts were made in the first month for populations A, B and F, so that in each of these the figure given for the second month is the grand total of new colonizers in the first two months after immersion. Furthermore it was not possible to make any observations in November, 1960, so that the figures for December are totals for the period October to December.

These data reveal several points concerning the ecology of *A. nigra.* First, it will be seen that with the exception of population E (immersion date June, 1960) all the populations had dense colonization during the first two months after immersion but very few colonizers in subsequent months. In the earlier paper the same phenomenon was observed but it had to be assumed that the fall-off in the number of new colonizers was due to some form of competition and was not due

TABLE I

The number of new individuals appearing in each month within different populations of Ascidia nigra. Months given in parentheses indicate the month in which the panels were first immersed

Month	Population					
	A (Oct., 1959)	B (Dec., 1959)	C (Feb., 1960)	D (April, 1960)	E (June, 1960)	F (Aug., 1960)
Dec., 1959	147					
Jan., 1960	10					
Feb.	2	463				
Mar.	0	4	199			
April	1	4	48			
May	1	2	7	415		
June	1	3	1	101		
July	1	1	0	5	74	
Aug.	6	0	0	2	12	
Sept.	1	0	13	2	0	
Oct.	6	0	33	0	0	187
Nov.	—	—	—	—	—	—
Dec.	8	2	37	35	80	31
Jan., 1961	2	11	6	5	34	4
Feb.	2	2		2	16	6
Mar.	1	1		4	1	2
April	0	2		1	6	8
May	0	0		30	2	5
June	0	0		8	3	1
July	0	0		3	0	0
Aug.	0	2		1	4	3
Sept.	1	3		0	7	2
Oct.	4	7		5	6	0
Nov.	4	7		9	9	9
Dec.	7	5		10	3	8
Jan., 1962	1	8		6	12	1
Feb.	2	1		3	1	2
Mar.	0	0		3	0	0

to the absence of larvae for settlement. The present data prove this assumption to have been correct. The fact that larvae were continually available for new colonization throughout the year is shown by the high density of colonizers occurring on the newly immersed panels of successive populations and yet, while these new settlements were occurring, the older populations were receiving a negligible number of new colonizers.

If we accept this view, that competition is responsible for the decline in new colonizers after the second month, we must enquire what form the competition takes and whether it functions by preventing new settlement or preventing growth of animals after metamorphosis. The development and succession in these communities have been briefly outlined elsewhere (Goodbody, 1963c) and will be discussed in detail in a later paper. The important aspects of this development are that an early *Balanus/Enteromorpha* community, in which *A. nigra* colonizes, is rapidly overgrown by the colonial ascidian, *Didemnum conchyliatum* (Sluiter), so that this ascidian and *A. nigra* become temporary dominants in the second seral stage. After a period of 7 to 12 months the *Didemnum* fragments and retreats, and sponges, lamellibranchs and stolidobranch ascidians become climax dominants. The rapid decline in colonizers after the second month is almost certainly due to the rapid spread of *D. conchyliatum* and, to a lesser extent, other colonial ascidians. These colonial ascidians overgrow and smother the smallest-sized young *Ascidia nigra* (Goodbody, 1963a) and also, by covering all available surface of the panel, effectively prevent any further settlement. Competition at this stage is therefore for space and not for food, those individuals of *A. nigra* which have successfully gained a foothold continuing to grow rapidly in association with the *D. conchyliatum*.

In the later stages of seral succession the problem becomes more complex and difficult to unravel. With certain exceptions noted below fresh colonization in the community continues at a low level even though the sheets of *D. conchyliatum* fragment and disappear. A new form of competition now arises from the climax dominants (*vide supra*) arising in the community.

I hope to show in later papers that the decline in the adult population of *A. nigra* is associated with the increasing dominance of these climactics and I believe that this is largely a question of competition for food from the more efficient stolidobranchs and sponges, while chemical effects from sponges may also be a contributory factor (Goodbody, 1961b). Support for the contention that competition for food is important at this stage is given by the observation that at all stages of growth *A. nigra* maintains its siphons projecting beyond the rest of the community and usually dies when this is no longer possible. This competition for food will also affect young settlers unless they establish themselves in peripheral portions of the community. Competition for space is of course also of great importance at this stage, as almost all available surfaces are now occupied. However, as the climax is approached the weight of material in the community increases and sloughing of small areas of the community occasionally takes place, exposing bare areas of panel. Sloughing of this sort is natural in old sessile communities (McDougall, 1943; Goodbody, 1963b) and effectively provides renewed conditions for seral succession. However, in the communities under study it was noted that unless the bared areas amounted to about 30 sq. in. (which was seldom the case) no new

TABLE II

The number of new Ascidia nigra appearing each month in all populations, in relation to the number of panels exposed. For details see text

Month	No. exposed panel sides	No. new colonizers	No. colonizers per exposed panel side
Jan., 1960	8	10	1.25
Feb.	8	2	0.25
Mar.	16	4	0.25
April	16	5	0.31
May	16	3	0.19
June	16	4	0.25
July	24	7	0.29
Aug.	24	8	0.33
Sept.	32	3	0.09
Oct.	32	6	0.18
Nov.	40	78	1.95
Dec.	40	78	1.95
Jan., 1961	40	56	1.40
Feb.	40	28	0.70
Mar.	40	9	0.22
April	40	17	0.42
May	32	11	0.34
June	40	12	0.30
July	40	3	0.07
Aug.	40	10	0.25
Sept.	40	13	0.32
Oct.	40	22	0.55
Nov.	40	38	0.95
Dec.	40	33	0.82
Jan., 1962	40	28	0.70
Feb.	40	9	0.22
Mar.	40	3	0.07

primary colonization occurred and the space was rapidly overgrown by adjacent sponges. Thus, we find that in these later stages of seral succession competition for both space and food, coupled with possible substances secreted by sponges, all combine to inhibit further immigration into the population of *A. nigra.*

Examination of Table I shows that in the months of November to January there is an increase in the number of new immigrants into the population, particularly in the first winter, 1960/61. This is best illustrated by expressing the data as the number of new immigrants per panel side exposed. In such an analysis the newly immersed panels must be excluded and hence only data from panels immersed for three months or longer are used. Data from population C are not used at all as the population suffered damage from an early stage. In May, 1961, data from population D are excluded as we know that the increased rate of colonization there was due to exceptional sloughing of the community. The remaining data are analyzed in Table II and Figure 1 and clearly illustrate the increased immigration rate in mid-winter. I have shown elsewhere (Goodbody, 1961a) that *Ascidia nigra* breeds throughout the year but has a maximum of reproductive activity and larval settlement in the winter months, and it seems tolerably certain that the increase in immigration into the population in November to January is the result of a massive increase in the number of larvae available, with a consequent increase in the number of ascidians successfully colonizing in the populations.

It will be noted that population E differs in two respects from other populations. First the initial colonization by 80 ascidians in the first two months was small, and second there was a large additional colonization of 114 animals in November/January, 1960/61. The panels on which this population developed were first immersed in June, 1960, and the small initial settlement is in keeping with earlier findings (Goodbody, 1961a) that the intensity of larval settlement is minimal at this time of the year. The high level of secondary colonization is due to the combined effects of the mid-winter abundance of larvae and an early retreat and fragmentation of the *Didemnum* community. It is usual in these communities for the *Didemnum* to be replaced by sponges immediately but, for reasons which cannot at present be explained, the *Didemnum* associated with population E retreated rather suddenly in November, 1960, and was not immediately replaced by sponge. As it fragmented and retreated it left fresh surfaces available for colonization by *A. nigra* which established itself before the *Didemnum* grew back or was replaced. This exceptional circumstance might be considered seriously to bias the figures given in Table II and Figure 1, so that it is necessary to point out that the total picture is unaffected by the data from population E. If we re-analyze the data in Table II for November and December, 1960, and January, 1961, excluding the data for population E, we arrive at the following figures for the number of colonizers per exposed panel side: November, 1.19; December, 1.19; January, 0.7. These figures still in-

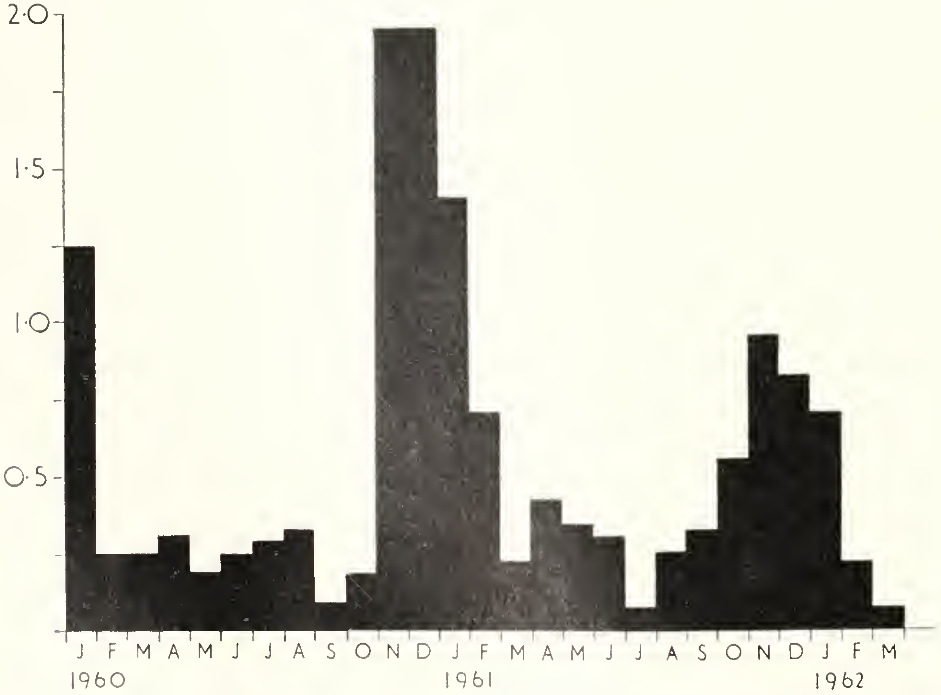

FIGURE 1. Colonization by *Ascidia nigra* in each month, expressed as the number of new individuals appearing per panel side exposed. For further details see text and Table II.

dicate a higher rate of immigration in the mid-winter months than at other times of year.

In conclusion the data presented in this paper demonstrate beyond doubt that *Ascidia nigra* is a primary colonizer in the sessile community in Jamaica. Settlement occurs on clean surfaces at a time when barnacles and filamentous algae are also colonizing. Subsequently a rapid development of the colonial ascidian, *Didemnum conchyliatum*, overgrows the panel surface, succeeding the barnacles and algae as the dominant organism in the community. Very small specimens of *A. nigra* are smothered by this growth (Goodbody, 1963a) which also inhibits any further settlement of the solitary species. Individuals of *A. nigra* of more than about two weeks old can survive the overgrowth and continue to grow in association with the *Didemnum*, so that at this stage it is a competitor for space and not for food which controls further immigration into the population. Later in the development of the community the *Didemnum* is replaced by sponges, lamellibranchs and stolidobranch ascidians, all of which may effectively compete for food with *A. nigra*. Thus, in the climax community inter-specific competition for food and space is responsible for preventing further colonization by *A. nigra*. A few specimens of *A. nigra*, however, succeed in colonizing at all stages of seral succession. This may occur either when sloughing of the community provides new surfaces for colonization or when larvae settle in peripheral positions in the community where competition for food is less intense.

SUMMARY

1. Artificial panels were used to allow six populations of *Ascidia nigra* to develop and grow naturally. The six populations started life at two-month intervals over a period of a year.

2. With the exception of a population starting life in June, all populations had dense colonization in the first two months after immersion of the panels, but few new colonizers in subsequent months.

3. The paucity of new colonizers after initial colonization is due to competition from other sessile organisms, first for space and later for food.

4. In the months November to December there is a marked increase in the number of new colonizers, irrespective of the age of the population. This is due to a large increase in the number of larvae available for settlement.

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STUDIES ON SPERMATHECAL FILLING IN AEDES AEGYPTI (LINNAEUS). I. DESCRIPTION[1]

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In a study on the female reproductive system of *Culex pipiens,* Kulagin (1901) illustrated for the first time a flask-shaped sac opening into the vagina, but he mistook it for the accessory gland. Not having seen Kulagin's study, Christophers (1923) clearly recognized that the accessory gland of mosquitoes was a separate structure from the dorsal diverticulum of the vagina and he termed this sac the *caecus.* Brelje (1924), apparently unaware of Christophers' work, pointed out Kulagin's error and described and illustrated the connections between the accessory gland, bursa copulatrix, spermathecae, and vagina in *Mochlonyx, Culex, Culiseta,* and *Aedes.* He termed the dorsal diverticulum of the vagina the bursa copulatrix. Brelje also discovered that the male mosquito deposited seminal material into this sac. Christophers (1960, p. 679) stated in his monumental work on *Aedes aegypti* that "the *caecus* . . . is a relatively small structure with the characters of a mucus gland," thus repeating Kulagin's confusion of two very different organs. In 1957 Burcham rediscovered both the structure and function of the bursa in *Aedes* and subsequently located Brelje's paper. Unfortunately Burcham's thesis was not published. Not having seen the earlier work, Hodapp *et al.* (1960) again rediscovered the structure and function of the bursa of *Aedes.* Some of this curious history was brought to light by Curtin and Jones (1961).

It has been known for a long time that female mosquitoes store sperm within from one to three spherical organs called *spermathecae* (Dufour, 1851), but it was not until 1957 that Burcham first explored some of the problems of how the sperm reach the thecae. He stated that a few sperm reach the storage organs within one minute after coitus, and he noted that few to numerous sperm were present within them in two minutes. He further remarked (p. 80) that the number ". . . steadily increased up to about five minutes after coitus" and that "the bursa copulatrix was essentially emptied within five minutes after mating." Schwartz (1961) found that sperm reached the thecae of *A. aegypti* between the 40th and 156th seconds after coitus. Spielman (1964), working with the same species, reported that sperm do not reach the thecae during the first 30 to 35 seconds after coitus but fill the organs in a period between 40 and 300 seconds.

It is the purpose of this paper to give a more detailed description of spermathecal filling in *A. aegypti* (Bangkok strain) than is currently available. A subsequent paper will deal with some of the physiological aspects of filling.

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Materials and Methods

The mosquitoes were reared in an insectary at 26° to 29° C. with a relative humidity ranging from 70% to 80%. The eggs were hatched in freshly boiled tap water that had reached room temperature and approximately 100 larvae were pipetted into a stender dish containing 250 ml. of water and a small pellet of Purina dog chow. When the larvae pupated, the pupae were sexed by placing them laterally on an ice cube and examining the pronounced differences in their external genitalia. The pupae were pooled in a beaker of water according to sex and placed in a one-cubic-foot screened cage so that the sexes would be completely separated at emergence. Adults had continuous access to 5% sugar water and the majority of the females were not offered a blood meal. Most of the studies were made on 3- to 7-day-old virgins. In the great majority of the experiments, the mosquitoes were forced to copulate by the technique of McDaniel and Horsfall (1957). Wheeler's modification (1962) of this technique proved essential to many of the observations required. Adults were generally anesthetized with nitrogen just before use. A fast-drying, non-toxic adhesive (Dekadhese) was applied to the head of Ward's #1 or #2 insect pin and gently but firmly pressed to the dorsal surface of the thorax. A series of males and females were thus arranged. The female-bearing pin was inserted into a cork glued to a microscope slide and the preparation placed under a dissecting stereo-microscope. The male-bearing pin was inserted into an adjustable holder allowing for gross and fine vertical movements. The male was moved up and down until his terminalium came into contact with that of the female at an angle of about 90°.

Dissections were made by grasping the thorax of the mosquito with sharpened Dumont #5 microforceps and the terminalium was placed into a small drop of buffered *Drosophila* saline (Ephrussi and Beadle, 1936). One finely sharpened needle was used initially to extract the organs to be studied. Further dissections, if needed, were made using two needles. Some extractions were made by using a second pair of microforceps to pull out the desired organs. Further experimental details are given in the text.

Results

1. *Efficiency of spermathecal filling after forced-copulation*

Virgins of the Bangkok strain force-copulated from 4 to 221 seconds and averaged 31.3 seconds with a standard error of 1.6 seconds (132 observations). In 18 cases which were allowed only one to five seconds of coital contact, only one was inseminated. In 8 cases which were allowed 9 to 10 seconds of coital contact, five (62%) were inseminated. In 23 cases which were allowed 15 seconds coital contact, 21 (91.3%) were inseminated.

Out of 737 cases of forced-copulation, the bursae in 8 females were observed to have what appeared to be only male accessory gland material and no visible spermatozoa. Three of these cases could be accounted for because the male used was found to be sterile and had no sperm cells available. Nevertheless, this agametic male copulated readily with 6 females in rapid succession. In two other cases, a male's freshly isolated terminalium had been used to copulate intact females. But three of the cases could not be accounted for, which presumably indicates that on rare occasions a normal male can ejaculate only accessory gland material.

Both sperm and male accessory gland material were present in all bursae of 41 (82%) of 50 females which were force-copulated and in all but one, sperm reached the spermathecae. Of these, 41.5% had sperm in all three thecae, 48.8% had sperm in two thecae, and 9.7% had sperm solely in the large median theca.

During the first 10 minutes after forced-mating, sperm moved about within the thecae of all females examined (28 cases; 44 thecae with sperm). In those females which were dissected 30 minutes to 6 hours after forced-mating, sperm moved in 30% to 62% of the thecae containing them (20 females; 56 thecae with sperm). Twenty-four hours after forced-mating, sperm were active in 80% of the thecae containing them (5 females; 10 thecae with sperm).

TABLE I

The extent and degree of spermathecal filling and motility of stored sperm in Aedes aegypti after different periods of unrestrained mating. Ten females used for each period

Time free coitus	Mean degree thecal filling	Large spermatheca			Lateral spermatheca			Lateral spermatheca			Bursa	
		No.	Degree	No. with motile sperm	No.	Degree	No. with motile sperm	No.	Degree	No. with motile sperm	No.	Condition
3 hrs.	2.70+	10	4+	3	10	3+	3	10	1+	3	10	Distended
6 hrs.	2.63+	10	4+	1	8 2	3+ 2+	2 2	9 1	1+ 2+	2 1	10	Distended
24 hrs.	2.70+	10	4+	8	10	3+	8	10	1+	10	10	Partially distended*
48 hrs.	2.57+	9 1	4+ 3+	10	10	3+	10	8	1+	8	3	Partillly distended**
72 hrs.	2.70+	10	4+	10	9 1	3+ 4+	10	6 2	1+ 2+	8	10	Empty

* Some sperm present in 50% of bursae; undulations of sperm seen in only one bursa.
** A few sperm seen in one bursa.

2. *Efficiency of spermathecal filling with cage-copulated mosquitoes*

Using Spielman's technique (1964), individual virgin females of the Bangkok strain were introduced into a 4 × 6 in. lantern chimney containing a number of unmated males, in order to obtain information on the time required for normal mating to begin, and to determine the time of coital contact under free mating conditions. The chimney was shaken to stimulate flight and copulation. In 5 cases, 13 to 32 seconds elapsed before copulation occurred (mean of 19.4 seconds). The mating time under these conditions ranged from 9.5 to 16 seconds, with a mean of 13.2 seconds. Ten seconds of coital contact under free mating conditions usually resulted in insemination. The mean coital time of 13.2 seconds is in close agreement with Spielman's value of 13.7 seconds for the Johns Hopkins strain of *A. aegypti.* Using larger cages, Roth (1948) and Burcham (1957) obtained a mean coital time value of about 16 seconds.

Three-day-old unmated males and females, 10 of each, were placed in each of five one-cubic-foot cages, and allowed to copulate freely for 3, 6, 24, 48, and 72 hours. The females were dissected and the condition of the bursae, the extent and degree of filling of the three spermathecae, and the motility of the stored sperm were noted. The degree of filling was qualitatively judged as follows: 4 +, numerous sperm; 3 +, many sperm; 2 +, few to many sperm; 1 +, very few sperm; and 0, no sperm detectable. The data for the five groups are summarized in Table I. Of the 50 females examined, 92% had sperm in all three thecae and 8% had sperm only in two thecae. Numerous sperm were in the large median theca in 98% of the cases, 94% had many sperm in one lateral theca, and 84% had a few sperm in the other lateral theca. One to 6 hours after free mating, sperm were actively moving in 26.7% to 33.3% of the thecae containing them (30 females; 72 thecae with sperm). After 24 hours, 86.7% of the thecae contained active sperm (10 females; 30 thecae with sperm). After 48 and 72 hours, sperm were active in all of the thecae containing them (20 females; 56 thecae with sperm).

Table II

Copulatory behavior and potency of a single six-day-old male Aedes aegypti when offered 12 virgin fenales in a 20-minute period

Virgin female number in order of presentation to the male	Seconds of coitus	Notes
1	10.2	Female escaped after coitus
2	13.6	Bursa full; 2 thecae with sperm
3	14.2	Bursa partially filled; 2 thecae with sperm
4	22.4	Bursa partially filled; 1 theca with sperm
5	10.8	Female dislodged the male; female not inseminated
6	68.0	Uninseminated
7	13.8	Female dislodged male; female not inseminated
8	25.8	Uninseminated
9	—	Male repeatedly attempted to clasp female's terminalium but aedeagus did not erect
10	22.8	Uninseminated
11	—	Male repeatedly erected aedeagus but did not establish good genital contact; uninseminated
12	—	Male erected aedeagus but only feebly clasped female's cerci and did not establish good genital contact

It is evident that data from forced-mating of virgins are significantly different from data from unrestrained or free mating between virgins. Thus, coitus lasts about twice as long with forced-mating, and the extent of insemination and thecal filling is more variable with forced than with unrestrained matings. Furthermore, sperm within the thecae become active more rapidly following forced-mating. The reasons and significance of such differences are not clear.

3. *Potency of individual males and spermathecal filling*

Each of five previously unmated males which were 6 days old were offered 4 to 12 virgin females in succession within a 10- to 20-minute period, using the forced-copulation technique. These males successfully copulated with 4 to 9 females and inseminated 3 to 5 of these. Data on one male which attempted to copulate with 12 females in a 20-minute period are shown in Table II. This individual was able to establish good genital contact for 10 to 68 seconds with 9 females, at least three of which he inseminated. When this male was subsequently dissected, his accessory glands appeared essentially like those of a once-mated male, but his seminal vesicles were shrunken and possessed only a few spermatozoa. As illustrated by the data in Table II, copulation can occur without insemination and even prolonged coitus (as with female #6) does not necessarily result in insemination. Erection of the male's aedeagus was not necessarily followed by copulation (as with females 11 and 12, Table II). In an earlier study (Jones, 1961), it was shown that when virgin males are allowed to copulate freely with a great excess of females in a cage for one hour, they inseminate about five females and their seminal vesicles are usually completely devoid of sperm and their accessory glands are greatly reduced in diameter and have little secretory material. This is in striking contrast to the repetitively force-mated males which only rarely get rid of all the seminal vesicle sperm and apparently ejaculate much less accessory gland material into the females. Conceivably, this difference in the amount of accessory gland material in the ejaculate could account for some of the differences already noted in the last section.

Two unmated males were presented to 6 virgins each and allowed to force-copulate with each one for 15 seconds, and the females were then dissected after 23 to 69 seconds. The first male inseminated 4 of the 6 females. The single, large, median spermatheca in all 4 of the 6 females had few to numerous spermatozoa; three of the females additionally had one of the lateral thecae with few to numerous spermatozoa. The second male inseminated 5 of the 6 females. Very few to numerous spermatozoa were found in the median theca in all 5 cases. Four of the females additionally had a few sperm in one lateral theca. In all of the above cases, the sperm in the thecae were inactive.

4. *Number of spermatozoa in the reproductive system*

Some preliminary estimates on numbers of spermatozoa were made on different portions of the male and female reproductive systems after forced-mating, using squashed whole mounts stained with aceto-lacto-orcein after Carnoy fixation. Sperm heads took the stain strongly and these were counted at a magnification of 970 × with the aid of an ocular grid. The inherent counting errors are considered large because the sperm often failed to spread evenly and this failure was especially evident with spermathecal squashes.

As shown in Table III, the terminal testicular chamber of one unmated male had approximately 700 spermatozoa. The terminal testicular chamber of three repetitively force-mated males had from 333 to 1209 spermatozoa (mean of 741.3). The sperm duct (vas deferens plus vas efferens) of one unmated male had 370 spermatozoa. The seminal vesicles of three unmated males had from 3700 to 6309 sperm (mean of 5132.3), while the seminal vesicles of two repetitively force-mated males had from 485 to 1374 sperm (mean of 929.5).

The bursae of 6 females which were freshly inseminated by a single highly potent male (he force-copulated in rapid succession with 7 females) were dissected within one to two minutes after each ejaculation. This one male ejaculated 254 to 2655 spermatozoa, and progressively fewer sperm were released with each succeeding forced-copulation (Table III). In sum, this male delivered 6126 sperm to 6 females and he did not inseminate the seventh female.

TABLE III

Numbers of spermatozoa in aceto-lacto-orcein squashes of different tissues of Aedes aegypti

Tissue	No. cases	Estimated numbers of spermatozoa
Terminal chamber of testis of unmated male	1	700
Terminal chamber of testes of repetitively, force-mated males	3	333; 682; 1209 (mean, 741.3)
Sperm duct of unmated male	1	370
Seminal vesicles of unmated males	3	3700; 5388; 6309 (mean, 5132.2)
Seminal vesicles of repetitively force-mated males	2	485; 1374
Bursa 1 to 2 minutes after:		
First ejaculation	3	1142; 1744; 2655 (mean, 1847)
Second ejaculation	1	554 (?)
Third ejaculation	1	1248
Fourth ejaculation	1	937
Fifth ejaculation	1	478
Sixth ejaculation	1	254
Bursa 1 to 1½ hours after first ejaculation	3	377; 584; 1142 (mean 701.0)
Bursa 1 to 1½ hours after second ejaculation	1	119
Spermathecae		
Large	4	141; 319; 325; 600 (mean, 346.3)
Small	4	68; 200; 250; 500 (mean, 254.5)
Thecae from 6 females after first ejaculation and 1–1½ hrs. after copulation	10	73-400 (mean 225.5)

The spermathecae of four females were dissected about one hour after forced-copulation with previously unmated males. Counts of spermatozoa in the large median theca varied from 141 to 600 (mean of 346.3), and counts of spermatozoa in one of the lateral thecae varied from 68 to 500 (mean of 254.5). Other counts made on spermathecae of unspecified size ranged from 73 to 400, with a mean of around 226.

The bursae of three females were dissected one to 1½ hours after forced-copulation with fresh unmated males. The number of spermatozoa that remained in the bursa after thecal filling had occurred was estimated to vary from 377 to 1142 (mean of 701) (Table III). These counts are considered to be much more reliable than direct spermathecal counts and suggest that of 1847 sperm (Table III) deposited in the bursa, about 1146 (62%) can reach the thecae. This, in turn, suggests that about 660 sperm reach the large theca and about 486 reach one of the lateral thecae. If these suggestions are correct, then the direct thecal counts of sperm are in error by a factor of about 2.

From these various estimations the following suggestions can be made. (1) The unmated male has about 5000 sperm available within his seminal vesicles. After rapid repetitive forced-mating, the male can ejaculate about 82% of the sperm within his vesicles. (2) The male ejaculates progressively fewer spermatozoa into each successive female. (3) Sixty-two per cent of the sperm initially deposited in the bursa reach the thecae, and 38% remain in this sac immediately after thecal filling.

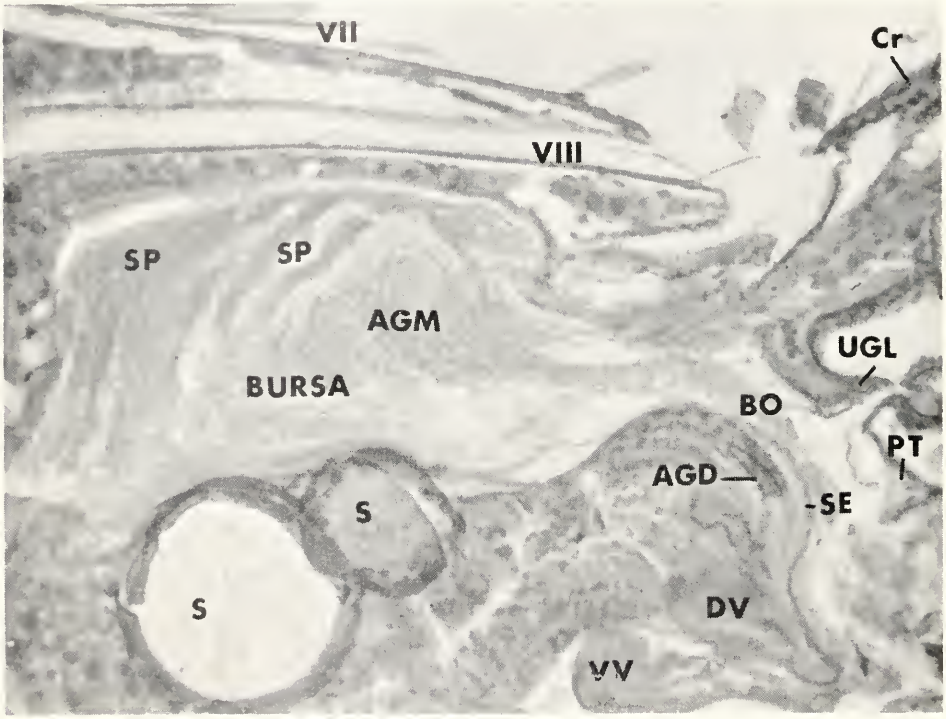

FIGURE 1. Sagittal section of *Aedes aegypti* female immediately after ejaculation of the male, showing the seventh (VII) and eighth (VIII) abdominal segments, cerci (CR), upper genital lip (UGL), bursal orifice (BO), accessory gland duct (AGD), spermathecal eminence (SE), dorsal valve (DV), ventral vaginal valve (VV), and spermathecae (S) of the female. Note the spermatozoa (SP) in packets in the dorso-anterior portion of the bursa and the large amount of finely granular male accessory gland secretion. The phallotreme of the male is shown at PT.

5. *The composition of the ejaculate*

The normal ejaculate consists of a relatively small amount of spermatozoa and a much larger amount of an acidophilic holocrine secretion of the male's accessory glands. Presumably, the spermatozoa are contained in a small volume of fluid within the seminal vesicles, and it is assumed that some of this seminal fluid is added to the ejaculate. When the ejaculate is seen under the dissecting microscope, it appears whitish; when viewed with a compound microscope, it has a greyish yellow cast.

The male accessory gland secretion includes a clear to finely granular material, round to ovoid granules of at least three different sizes, a few free nuclei, and even some intact, round to ovoid accessory gland cells of various sizes with large granular inclusions. If the accessory glands are ruptured in an open drop of saline, the exuding material does not usually vacuolate and the cells, free nuclei, and granular inclusions do not ordinarily lyse, although the cells may become swollen. The accessory gland secretion in an open drop of saline forms a dense, viscous, sticky mass which will rapidly clog a micropipette. However, if the saline mount is quickly covered with a layer of immersion oil before the glands are ruptured, then the exudate can be generally drawn into a micropipette without clogging.

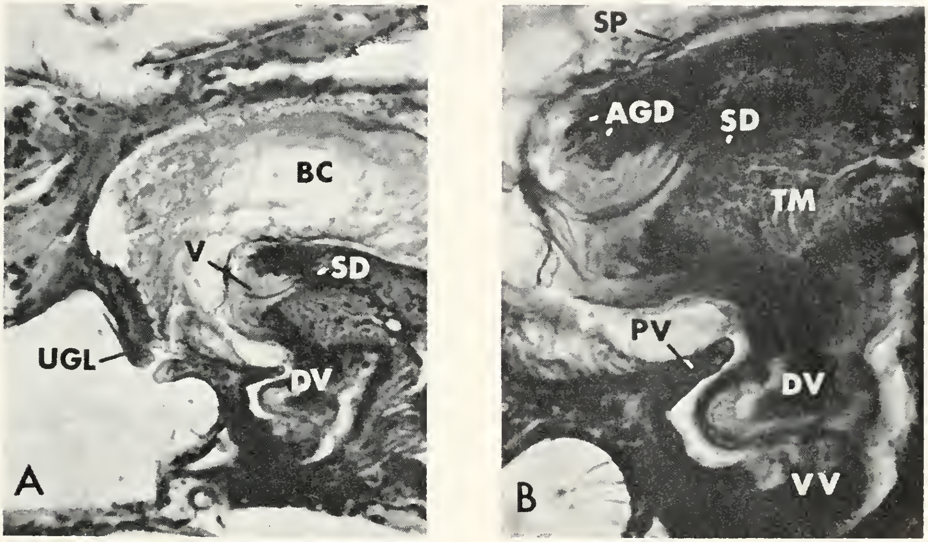

FIGURE 2. Sagittal section of *Aedes aegypti* female during spermathecal filling. In A is shown the upper genital lip (UGL), the swollen bursa (BC), the vestibule (V), the spermathecal duct (SD), and the dorsal vaginal valve (DV). B is the same section, at greater magnification, showing the sperm (SP) assembled in packets on the ventral floor of the bursa, and sperm making the U-turn into the vestibule. Note the position of the accessory gland duct (AGD), one spermathecal duct (SD), the posterior vaginal valve (PV), dorsal vaginal valve (DV) and ventral valve (VV). The transverse muscles of the spermathecal eminence are shown at TM.

6. *Some changes in the ejaculate within the bursa*

At the very moment of deposition, the locomoting packets of sperm are ejected dorsally above the accessory gland secretion (Fig. 1). In sagittal sections, the freshly inseminated bursa measures about 300 to 350 μ in length and varies from 78 to 100 μ in depth. The bursal orifice measures from about 18 to 25 μ. The spermatozoa measure about 250 μ (Christophers, 1960). While most of the sperm rapidly spread to the edges of the bursa, some of them become trapped in the granular portion of the accessory gland secretion. As Spielman (1964) has pointed out, many sperm rapidly assemble on the ventral wall of the bursa facing the orifice (Fig. 2B, SP). Those sperm at the blind anterior end of the bursa tend to remain quite active for about 17 minutes in oil-covered drops of saline, after which they tend to become noticeably less visible and less active. Those sperm at or near the bursal orifice tend to be especially active.

Thirty seconds after ejaculation, the bursal wall in sagittal sections measured 2.2 to 3.3 μ. Two to three minutes after insemination, the bursal wall was greatly swollen (7.5 to 12 μ thick), hyaline, and the cells had large colorless vacuoles. The bursal wall was swollen and vacuolated for at least one hour after insemination. In fresh unstained whole-mounts of freshly inseminated bursae, we have seen what appeared to be a very delicate membrane surrounding the seminal mass, but no such membrane was visible in any of the histological sections.

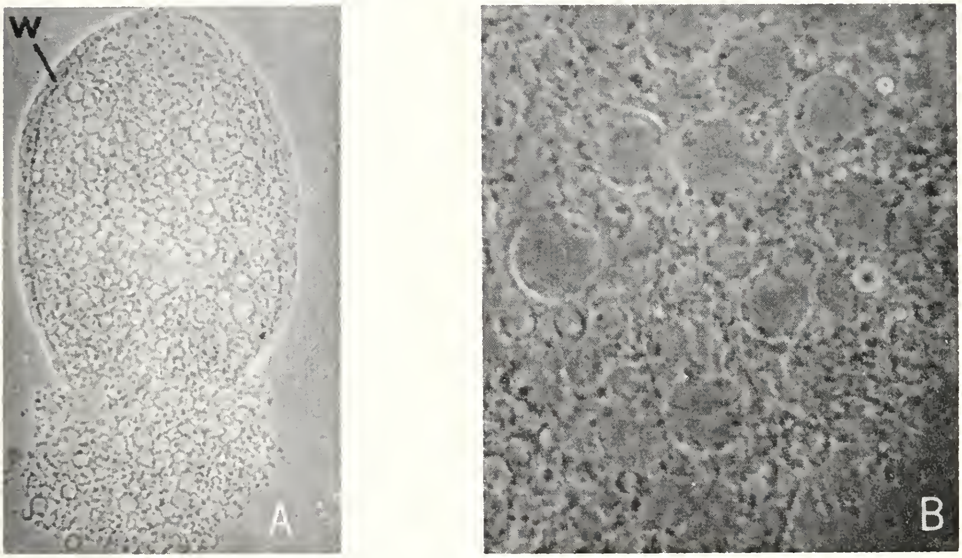

FIGURE 3. Unstained saline whole-mount dissection of the bursa of *Aedes aegypti* about 10 minutes after insemination, showing the fully vacuolated ejaculate. Note the swollen wall (W) in A and, at greater magnification (B), the large vacuoles and granules within the ejaculate. The largest vacuole in B is about 20 microns in diameter.

Three to five minutes after insemination, large vacuoles first appear within the granular portion of the ejaculate and they steadily increase in number until the bursal contents become filled with vacuoles within a granular matrix (Fig. 3). The large vacuoles measure about 20 μ in diameter. These vacuoles are very clear in whole mounts but may be indistinct in sectioned material. The ejaculate may be fully vacuolated within about 10 minutes at 27° C. At 36° to 37° C., the bursal contents fully vacuolate in about one minute. The completely isolated, freshly inseminated bursa will fully vacuolate in a drop of saline covered with a layer of immersion oil. When a male's accessory glands were crushed in the vicinity of the freshly dissected virgin bursa, the glandular exudate did not vacuolate. If the female is hosed with carbon dioxide for 5 minutes three seconds after coitus, the seminal material within the bursa does not vacuolate.

In many preparations in which the bursal contents were vacuolating or had already fully vacuolated, the sperm were not visible through the intact wall of the bursa, but when the bursa was opened, sperm were found. Bursal sperm retain a highly variable amount of undulatory activity for about 6 hours and sometimes for as long as 24 hours after insemination. In a number of cases, however, the sperm within the bursa were mostly inactive within about 75 minutes after ejaculation.

Bursae filled with vacuolated seminal material were observed up to 6 hours after insemination. Twenty-four hours after insemination, the bursae were partially distended; the wall was no longer thickened and vacuolar, and the contents of the sac were finely granular, had a yellowish brown cast, and were devoid of any vacuoles.

The bursa was never observed to contract in saline or oil-covered saline preparations of either uninseminated or inseminated *A. aegypti*. Histological sections showed the bursa to be completely devoid of muscles.

7. *Structure of the spermathecae and their ducts*

The median spermatheca of *A. aegypti* measures about 100 μ in diameter, and each lateral theca measures approximately 75 μ in diameter. The thecae are completely devoid of muscle and were never observed to contract in any type of preparation examined. When the spermathecae of virgins are opened under a deep layer of oil, no bubble escapes. When the thecae are crushed in a drop of xylene containing sudan black, a colorless halo of fluid appears immediately around them. This colorless watery fluid within the thecae did not react with phenol red, neutral red, sudan III, British Drug House Indicator, or with Hydrion papers.

Although the spermathecal ducts are covered by a single layer of evenly spaced circular muscles (Curtin and Jones, 1961), we have never seen these ducts contract in saline or oil-covered saline whole-mounts. The spermathecal ducts, when stretched out in saline, measure about 265 μ in length, and the clear lumen of these varies from about 2 to 3.5 μ.

8. *Speed of sperm transfer*

Twenty-two females were used to study how rapidly bursal sperm could reach the thecae after forced-mating. The females copulated with males for 9 to 54 seconds and were immersed in liquid chloroform 2 to 45 seconds thereafter. Three of the females did not become inseminated, although the males had copulated with them for 9 to 25 seconds. Eight of the females were killed 2 to 24 seconds after coitus and in three of them (37.5%), sperm had reached the thecae. In the first of these three cases, the female (which had copulated for 53.8 seconds and was killed in 18 seconds) had only a very few sperm in two thecae. In the second case, the female (which had copulated for 28 seconds and was killed in 20 seconds) had many sperm in the large theca and a few sperm in one lateral theca. In the third case, the female (which had copulated for 15 seconds and was killed in 24 seconds) had only a few sperm in the large theca. In the five other cases, however, sperm were found only in the bursa and none reached the thecae. Eight out of the 22 females were immersed in chloroform 30 seconds after coitus and were then dissected. In only one of these females had sperm reached the large median theca. The last three females were killed 40 to 45 seconds after forced-mating, and in all three cases many sperm had ascended to the thecae, and in two of the females sperm were in two thecae. Thus, following uninterrupted forced-coitus, while a few sperm apparently are capable of reaching the thecae of a few females as early as 18 seconds after copulation, in most cases the sperm begin to fill the thecae between 30 and 45 seconds.

That highly variable results can be obtained is shown by the following data. Coitus of 7 pairs was permitted for exactly 15 seconds, after which the females were killed at 10-second intervals from 30 to 90 seconds after forced-mating, by exposing them to very strong ether fumes. Only a few sperm reached two thecae after 30, 40 and 50 seconds. Many to numerous sperm were found in two thecae after 60, 70, 80 and 90 seconds.

In spite of the variability of the data, it is evident that spermathecal filling in *A. aegypti* is a rapid event, and we are much inclined to agree with Burcham (1957) and the data presented by Spielman (1964) that no transfer occurs after the first 5 minutes following coitus.

9. *Histological observations on the reproductive tract of inseminated females before, during and following spermathecal filling*

Spielman (1964) stated (p. 341) that ". . . 5 minutes after coitus, sperm were scarce in the anterior portion of the . . . bursa" and that one hour after coitus "the bursa was filled with coarse material and the remaining sperm were compressed into the posterior end and into the upper atrium." Our observations are not in agreement with these statements. The bursae of 22 mosquitoes were dissected 23 minutes to 6 hours after forced-copulation and in all cases many to numerous sperm were still present in the distended bursae. Dr. Spielman has kindly permitted us to examine the sections which he prepared for his studies on spermathecal filling after free mating. Our observations on his material follow.

Sections made 30 seconds following coitus showed that the bursa was distended with ejaculate, the majority of the sperm being antero-dorsally located, but at least one dense packet of sperm was seen on the ventral wall of the bursa at its orifice. No sperm were observed in either the atrium, vestibule, thecal ducts, spermathecae, or common oviduct. The ventral tuft just inside the female's ventral genital lip slanted dorsally into the bursal orifice but did not block this opening. In some sections, the posterior valve of the vagina was pressed against the surface of the dorsal valve, but in other sections a gap of variable dimensions was seen between these two vaginal valves. The ventral valve in some sections was pressed against the dorsal valve.

Thirty-five seconds after coitus, sections showed some male accessory gland material within the lumen of the upper vagina, but no sperm were detected. Numerous sperm were still in the dorsal portion of the bursa and at its blind anterior end. Many sperm in the bursa were seen in ventrally-directed arcs. Dense packets of sperm were seen making a sharp U-turn from the bursal orifice into the spermathecal vestibule (see Fig. 2). The heads of these sperm were in contact with the intima of the spermathecal ducts. No sperm were visible in the upper vagina, thecae or common oviduct. The ventral tuft slanted upward into the bursal orifice, blocking the center of it about half way. The spermathecal eminence appeared to be elevated and the vestibular opening seemed shifted into a position dorsal to the ventral tuft. The posterior vaginal valve in some sections was pressed against the dorsal valve.

Sections made 43 to 60 seconds after insemination of the bursa showed sperm, and, in one case, male accessory gland material, free in the lumen of the upper vagina (Fig. 2). Many sperm were flattened against the surface of the dorsal vaginal valve. No sperm were visible in the lower vagina or in the common oviduct. The bursa was distended, and many sperm were ventrally aligned in dense packets at the bursal orifice. The median ventral tuft slanted halfway across the bursal orifice. The spermathecal eminence still appeared elevated. Many sperm were seen making the U-turn into the vestibular opening (Fig. 2). Sperm were observed inside two spermathecal ducts, and a few sperm were seen inside the spermathecae. The posterior vaginal valve in some sections touched the dorsal valve.

Ten minutes after insemination, sections showed sperm still present in the upper vagina, mostly flattened against the dorsal valve. No sperm were visible in the lower vagina or the common oviduct. Numerous sperm were still present in the bursa and many of them were aligned on the ventral wall at the orifice. The ventral tuft appeared to completely block the vestibule in one series of sections made at 10 minutes.

In sections made one hour after coitus, sperm were still within the upper vagina, free in its lumen, and against the dorsal valve; and for the first time, sperm were seen in the lower vagina and in the lumen of the common oviduct. Numerous sperm were still observable throughout the distended bursa. The vestibule appeared fully open to the bursal orifice. The ventral tuft did not block the vestibule. Sperm were still detectable within the spermathecal ducts and within two thecae. Sperm were especially dense at the entrance to each of two thecae.

According to Spielman (1964, p. 341), the common oviduct contains ". . . masses of agglutinated immobilized sperm" one hour after coitus. Dissections made on the Bangkok strain after forced-copulation showed a few undulating sperm within the common oviduct at 57 to 69 minutes in three females, but no sperm were visible in the oviducts of two other females. Two hours after forced-copulation, one female had undulating sperm in her common oviduct as far as the ampullae, but no sperm were seen in the oviduct of another female dissected at the same time. No sperm were visible in the common oviduct of one female dissected 6 hours after forced-copulation.

Our observations indicate that in both freely-mated and force-mated *A. aegypti* many sperm remain within the bursa following spermathecal filling, and that most of them are not concentrated at the bursal orifice. It is of considerable interest that the female has no anatomical device that could account for the fact that those sperm which are aligned at the open bursal orifice no longer attempt to reach the spermathecal vestibule. Even sperm which are free within the lumen of the upper vagina are no longer oriented towards the vestibule after spermathecal filling.

Numerous dissections were made on force-mated and freely-mated specimens before, during, and following spermathecal filling, and in not one case were sperm ever detected within the spermathecal ducts. It is difficult to reconcile this with the presence of a few sperm in these ducts in sectioned material made one hour after free-mating. It is possible that during dissection the sperm within the ducts quickly entered the spermathecae.

10. *The behavior of spermatoza*

Observations were made on the activity of spermatozoa within various portions of the unmated male's intact reproductive system in oil-covered saline whole-mounts. The spermatozoa often exhibited intense whirling or spiralling activity within the posterior testicular chamber. They were either inactive or showed a highly variable degree of activity within the sperm ducts, and sometimes undulated within the seminal vesicles. Sperm retained activity within the testes for 54 to 270 minutes and undulated in some seminal vesicles for 60 to 348 minutes. Sperm within the sperm ducts, if active at all, were generally active for less than 38 minutes and for not more than 196 minutes.

TABLE IV

Changes in motility of sperm with time in thecae isolated from force- and freely-mated Aedes aegypti one hour after insemination. Ten females used for each of the two groups

Time after isolation of thecae	% Thecae with moving spermatozoa	
	Force-copulated*	Freely mated**
less than 10 mins.	46.4	33.3
1 hour	85.7	58.3
2 hours	64.3	8.3
3 hours	42.9	0
4 hours	10.7	0
5 hours	3.6	0

* Twenty-eight thecae contained sperm.
** Twelve thecae contained sperm.

The spermathecae of force-mated and freely-mated mosquitoes were isolated one hour after copulation in a drop of saline and the preparation covered with a layer of immersion oil so that the activity of the sperm within the thecae could be noted at hourly intervals. As shown in Table IV, sperm were generally more active and remained active for a longer period in the force-mated than in the cage-mated group. While no activity was observed three hours after isolation of the thecae from the cage-mated mosquitoes, sperm were actively moving in 42.9% of the thecae of the force-mated females at this time.

The following types of activity were observed in spermatozoa released from seminal vesicles into a drop of saline covered with a layer of immersion oil: (1) very rapid locomotion with the short, sharp, thin, stiff, needle-like head piece tilting up and down as the long thin tail made rapid, large wave undulations. These explosive progressive locomotory movements occurred either in a generally straight line in any direction, or the sperm would circle about briefly. In saline drops which were not covered with a layer of oil, locomotions were only rarely observed. (2) Very rapid and intense coiling or lashing motions of the tail were observed, especially when the sperm occurred in clusters and when the head was at an interface. Many times the tails of clustered sperm whirled or lashed synchronously. (3) Slow, smooth, regular, large wave undulations of the tail were observed when sperm were congregated at the saline/oil interface. (4) Irregular undulations or oscillations of highly variable amplitudes were observed in sperm which had ceased locomoting or which had stopped the smooth regular undulations. Different portions of the tail were capable of undulating at very different rates and with different amplitudes. The waves moved away from the head piece.

Observations were made on the activity and survival time of sperm released from the seminal vesicles. Highly variable results were obtained, depending upon the technique and the location of the sperm within the preparation. When the seminal vesicles were ruptured into an open drop of saline, many sperm which reached the edge of the drop quickly lost their motility in one to three minutes. Those sperm which did not reach the edge of the drop undulated irregularly and lost all activity fairly rapidly. However, those sperm still inside the torn vesicles tended to remain active for about four minutes in open saline drops. When the vesicles were ruptured in a very small amount of saline that had been covered first with a drop of immersion oil, the sperm were often intensely active for two to 15 minutes, especially around the surface of the vesicles. Generally, the sperm in such preparations lost all activity in three to 87 minutes after release. Those sperm which moved out into the layer of oil very quickly ceased moving. When the vesicles were opened in a moderate-size drop of saline that had been covered with a layer of immersion oil, those sperm which were strongly oriented at the saline/oil interface remained active for 16 to 60 minutes, but those which did not reach the edge of the drop tended to lose their activity in about one minute. The sperm inside the seminal vesicles remained quite active for two to 119 minutes (in most cases for about 6 minutes); highly variable numbers undulated feebly for 182 to 328 minutes. Sperm on the outside of the vesicles tended to have their heads oriented to the vesicles' surface and were often very intensely active for about four minutes.

In coverslipped saline mounts in which air bubbles had been trapped, the sperm head was frequently strongly oriented to the saline/air interface.

To study whether chemotaxis might be involved in sperm migration, seminal vesicles and bursal sperm were released in the vicinity of intact male accessory glands, male accessory gland exudate, and onto freshly excised vaginal tissues. In many cases, the tip of the sperm head was strongly oriented to all of these tissues, but sperm did not specifically congregate around them. The head of seminal vesicle and bursal sperm did not become specifically oriented to fat body, testes, somatic muscles, bursa, female accessory gland, or its duct, spermathecal ducts, intact or crushed spermathecae, common oviduct, or even to a freshly laid egg.

To determine whether the sperm head would be oriented in or against the direction of a moving stream, the intact seminal vesicles were dissected into a drop of saline on a glass slide next to a long rectangular coverslip supported on two sides by capillary glass rods. The thin space between the coverslip and slide was filled with saline. The seminal vesicles were cut open so that the sperm poured out into the saline just under the edge of the coverslip and a strong current was produced by withdrawing saline at the other end. In all such preparations, the sperm heads were precisely aligned in the direction of the flowing stream of saline. Dead sperm were not precisely aligned. This type of rheotaxis of live *Aedes* sperm is exactly the opposite of that of bull or human sperm which are known to orient the head piece against the direction of a moving stream (see, *e.g.*, Rothschild, 1962).

Thus, the head of the spermatozoön of *A. aegypti* becomes oriented toward certain tissues and becomes aligned in the direction of a flowing stream, and the tail piece is capable of propelling the cell rapidly.

We are indebted to Dr. Andrew Spielman of Harvard University for allowing us to examine his histological sections and for many stimulating discussions. Our Figures 1 and 2 were taken from material which Dr. Spielman loaned us. We are most grateful to Mr. Kenneth W. Ludlam for his help with many of the experiments. Useful suggestions concerning the manuscript were made by Drs. Norman T. Davis, Arden O. Lea, P. T. M. Lum and A. Glenn Richards, and by Elizabeth D. Jones.

Summary

1. With the forced-copulation technique, the Bangkok strain of *Aedes aegypti* can ejaculate within the first five seconds of coitus but usually does so within 10 to 15 seconds. The male force-copulates for 31.3 seconds, and 82% of the females become inseminated. In 90% of them, spermatozoa reach two of the three thecae. With naturally-mated mosquitoes copulation is significantly shorter in duration, all of the females become inseminated and in 92% of them spermatozoa reach all three thecae.

2. The terminal chamber of the testis of an unmated and repetitively force-copulated male has about 700 spermatozoa. Each sperm duct has about 370 sperm. The seminal vesicles of unmated males have about 5000 spermatozoa, while the vesicles of repetitively force-mated males have about 930.

3. With rapid repetitive force-copulation, the male ejaculates progressively fewer spermatozoa into the bursa of each successive female. Sperm counts made on 6 ejaculates from one male varied from 254 to 2655.

4. Counts on spermatozoa remaining in the bursa after spermathecal filling indicate that 62% of them leave the bursa, and suggest that about 660 sperm reach the large theca and 486 fill one of the lateral thecae.

5. Most of the sperm deposited in the bursa quickly spread to the edges of the sac, and many become aligned on its ventral wall. The wall of the bursa greatly swells two to three minutes after insemination. Shortly thereafter the accessory gland secretion within the ejaculate begins to vacuolate and may be fully vacuolated within 10 minutes and remains vacuolated for at least 6 hours.

6. With forced-copulation, a few sperm may be capable of reaching the thecae within 18 seconds but in most cases sperm begin to reach the thecae between 30 and 45 seconds after coitus. Complete thecal filling can occur in 90 seconds and probably is terminated within the first five minutes or less after coitus. Fol-

lowing spermathecal filling many active sperm remain in the bursa for some time. Following spermathecal filling those sperm at the bursal orifice no longer make the U-turn towards the open spermathecal vestibule.

7. Spermatozoa within the isolated but intact male reproductive system may remain active for five to six hours in oil-covered saline whole-mounts. Spermatozoa released from seminal vesicles in oil-covered saline drops exhibit four types of movement: (a) brief, rapid, explosive, progressive locomotion, (b) rapid synchronous coiling when the cells are in dense clusters and the head is at certain interfaces, (c) smooth undulations *in situ,* and (d) irregular undulations or oscillations. The heads of sperm of *Aedes* become oriented in the direction of a moving stream.

8. Sperm released from the seminal vesicles may become strongly oriented toward the male accessory glands and its exudate, and to freshly excised vaginal tissues, but they do not specifically congregate about these tissues in oil-covered saline whole-mounts. Seminal vesicle sperm do not become oriented to freshly excised fat body, testes, somatic muscles, bursa, female accessory gland or its duct, spermathecae or their ducts, ovary, common oviduct, or a freshly laid egg.

Conclusions

Many sperm deposited in the bursa of female *Aedes aegypti* (Linnaeus) rapidly locomote around the male accessory gland secretion of the ejaculate and assemble on the ventral floor of the sac at its orifice where they undergo rapid and violent synchronous coiling movements. The strong orientation of the sperm head to certain interfaces presumably guides the long, thread-like contracting cells over a U-shaped route directly into the vestibule where they first contact the opening of the spermathecal ducts. Bundles of sperm swiftly ascend the ducts, presumably only in female fluids, and simultaneously reach two or three thecae. Shortly after sperm begin to fill the already fluid-filled thecae, the bursal wall swells and presumably secretes material into the ejaculate. After this the accessory gland secretion of the ejaculate begins to vacuolate, and a short time after this, the still active sperm at the open bursal orifice stop moving into the vestibule.

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NEW SPECIES OF ACOEL TURBELLARIANS FROM THE PACIFIC COAST

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The acoel turbellarians have been accorded very little attention in North America. Only a few species have been described, and some of these will have to be investigated more completely before their taxonomic position can be established with any degree of certainty. On the Pacific coast, where acoels are abundant in bottom sediments, on algae, and in other situations, apparently only two species have been named. One of these is *Polychoerus carmelensis* Costello and Costello (1938), from central California; the other is *Childia groenlandica* (Levinsen), an acoel which has a wide distribution in North Atlantic waters and which has recently been reported from San Francisco Bay, California (Hyman, 1959).

During the summers of 1961 and 1962, and the autumn of 1964, several species of acoels were taken at various localities on San Juan Island, in the San Juan Archipelago, Washington. Mature individuals of three of these were recovered in sufficiently large numbers to permit a thorough study of their morphology and description as new species. Additional material of one of these acoels was found at Charleston, Coos County, Oregon, during the summer of 1964.

The acoels are a difficult group with which to work. Most of them are small, and certain of their syncytial structures are not sharply delimited. Many published descriptions are incomplete and poorly illustrated. There are even some rather detailed accounts which do not focus sharply on pertinent details, and from which one cannot form a clear picture of the morphology of the acoels concerned. In deciding the taxonomic position of the new species described here, I have relied upon the summary of the genera and higher taxa of acoels given by Westblad (1948).

I wish to express my appreciation to Dr. Robert L. Fernald, Director of the Friday Harbor Laboratories, for many courtesies which facilitated my work.

METHODS

The acoels described in this contribution were for the most part obtained by taking up masses of green algae (*Ulva* and *Enteromorpha*) and washing them by agitation in a pail of sea water. A thin layer of sediment (consisting largely of muddy sand from the substrate) obtained in this way was then distributed in large culture dishes, in water about 2 or 3 cm. deep. Samples of sediment sucked up with a large pipette and examined with a dissecting microscope often contained acoels,

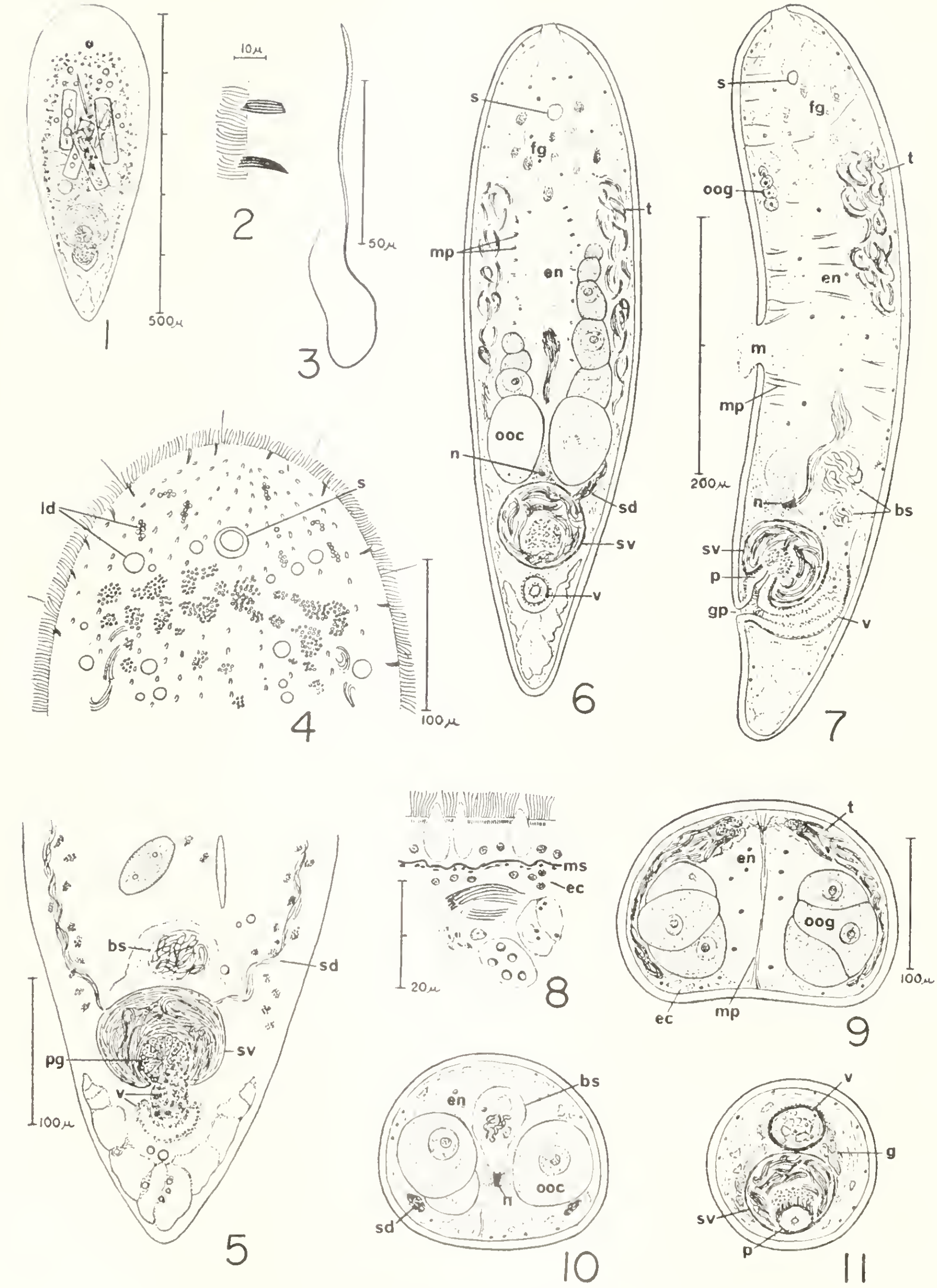
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Plate I—*Parotocelis luteola*

All figures were prepared with the aid of a camera lucida, but in the case of specimens drawn from life (Figs. 1–5), most details were sketched in free-hand. Figures 6 to 11 are based together with rhabdocoels, alloeocoels, copepods, amphipods, and other small organisms.

My descriptions are based entirely on sexually mature specimens examined or fixed soon after collection. (Worms which are immature or which are not well nourished are unreliable.) The acoels were studied extensively in life, in both transmitted and reflected light. Gentle compression of the worms under a coverglass was usually necessary to make certain structures clearly visible. Addition of a small amount of a solution of magnesium chloride (approximately isotonic with sea water) to the drop of water in which the worms were swimming was generally helpful in narcotizing them in an extended condition without obviously affecting their appearance.

Stained whole-mount preparations were less useful than living acoels for morphological studies. However, a few whole-mounts of worms fixed in Bouin's fluid and stained with alum hematoxylin or borax carmine were prepared for permanent records.

The morphology of the acoels described in this paper was worked out largely by study of transverse, sagittal, and frontal serial sections (6 μ or 8 μ). The worms were fixed, usually after being narcotized, in Bouin's fluid or in a mixture of 90 ml. of a saturated aqueous solution of mercuric chloride with 10 ml. of formalin and 5 ml. of acetic acid, and then embedded in paraffin. Iron hematoxylin was used routinely for staining; sometimes the preparations were counterstained with eosin, orange G, or fast green FCF. A few series were stained with Harris' alum hematoxylin and eosin.

DESCRIPTIONS OF SPECIES

Parotocelis luteola gen. nov., sp. nov.

Most of my material of this acoel was taken in shallow pools at the margins of a body of water known locally on San Juan Island as Argyle Lagoon (Lat. 48°

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on sections (6 μ) of specimens fixed in Bouin's fluid and stained with iron hematoxylin; certain details were supplied from adjacent sections in the same series.

Abbreviations for all figures: b, brain; bs, seminal bursa; ec, ectocytium; en, endocytium; fg, frontal glands; g, glands surrounding copulatory organs; ga, genital atrium; gp, genital pore; ld, lipid droplets; m, mouth; mp, parenchymal muscles; ms, subepicytial muscles; n, nozzle of seminal bursa; ooc, oocyte; oog, oogonium; p, penis; pg, granule-filled masses at tip of penis; s, statocyst; sd, sperm duct; sv, seminal vesicle; t, testis; v, vagina.

FIGURE 1. Specimen in contact with substrate; dorsal view.

FIGURE 2. Rhabdites at left margin of body.

FIGURE 3. Mature sperm.

FIGURE 4. Anterior end (specimen slightly compressed under coverglass); dorsal view.

FIGURE 5. Posterior end (specimen slightly compressed under coverglass); dorsal view. The anterior portion of the vagina, in which the lumen is obliterated by a syncytium containing refractile granules, obscures part of the seminal vesicle beneath it.

FIGURE 6. Frontal section. One of the oocytes is undergoing a maturation division.

FIGURE 7. Median sagittal section.

FIGURE 8. Epicytium, epicytial glands, subepicytial musculature, and portion of testis in transverse section just anterior to mouth.

FIGURE 9. Transverse section just anterior to mouth.

FIGURE 10. Transverse section through seminal bursa of same specimen.

FIGURE 11. Transverse section through penis, seminal vesicle, and anterior portion of vagina of same specimen.

31.3′ N.; Long. 123° 0.6′ W.).[1] The worms are usually abundant in muddy sand supporting a growth of *Enteromorpha*. *P. luteola* has also been collected in small numbers at Friday Harbor, among *Ulva and Enteromorpha* growing on a substrate of gravel mixed with muddy sand, at tide levels of about 0 to +2 ft.

When gliding actively, the length of *P. luteola* is typically about two and a half times the width (Fig. 1). The greatest width is usually near the end of the first one fifth of the body. The anterior end is broadly rounded; posteriorly, the body tapers rather gradually to a nearly acute tip. The largest specimens are about 700 μ in length and 230 μ in width, but they may become extended temporarily to a maximum length of about 840 μ and a width of about 180 μ. In worms which are in tight contact with the substrate, the thickness in the mid-dorsal region is usually about one-half the greatest width, so that the body appears definitely flattened. When swimming free, the worms become nearly cylindrical.

The statocyst (Fig. 4) lies about midway between the ventral surface and the dorsal surface, near the end of the first one-tenth of the body. In larger specimens, the diameter of the statocyst is about 20 μ, and that of the statolith is about 12 μ. Viewed on edge, the statolith is nearly hemispherical; the convex surface is uppermost.

In reflected light, the coloration of the body as a whole, excluding the digestive endocytium, is whitish, tinged faintly with orange; the orange color becomes more pronounced anterior to the statocyst. In most specimens which contain ingested diatoms, the endocytium is green, although in an occasional individual this region is brown or yellowish brown. In the area just behind the statocyst, there are small masses of a material which appears white in reflected light. Three discontinuous longitudinal streaks of this material extend for some distance posteriorly.

In strong transmitted light, the body is more or less translucent. In the endocytium, freshly-ingested diatoms with their characteristic olive-green, yellow-brown, or yellow-green color may be seen, together with those which have turned green or bluish green. Chlorophylls diffusing out of the diatoms undergoing digestion appear to be responsible for the green or blue-green color typically observed in this general area. In the ectocytium anterior to the statocyst, there is a crescentic band of yellowish-orange pigment; similar pigment is often found in small areas in the posterior part of the body, around the copulatory organs. Lipid droplets are scattered throughout the body. The larger of these, which may attain a diameter of about 20 μ, are yellow or orange; the smaller ones may be nearly colorless, yellow, orange, or bluish green, or some mixture of these colors. The lipid droplets apparently concentrate pigments which diffuse out of the diatoms. The material which is white in reflected light is seen to consist of granules or rods of a refractile substance. These granules form small aggregates (Fig. 4) within the ectocytium in the dorsal part of the body just behind the statocyst, and dorsal and dorsolateral to the endocytium. Under low magnification, the aggregates appear nearly black; under higher magnification, they appear brown. However, the individual bodies in the aggregates are nearly colorless.

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[1] Argyle Lagoon is not natural. It was formed after an accumulation of gravel from a hillside excavation became deposited, together with sand and debris, on the seaward side of a small inlet of North Bay. At high tide, water may enter the lagoon through its narrow connection with the inlet. At low tide, some water drains out of the lagoon. However, the water level in the lagoon does not vary a great deal, and most of the time it is higher than that of North Bay and the inlet.

The body is entirely covered by cilia about 10 μ long. Scattered over the body surface there are stiff, cilia-like bristles (Fig. 4) up to about 24 μ long. These are probably sensory in function. The bundles of epicytial rhabdites (Fig. 2) are rather evenly distributed on both the dorsal and the ventral surfaces. They fall into poorly-defined short rows (Fig. 4).

The mouth is located on the ventral surface, slightly anterior to the middle of the body. The pore is kept closed most of the time, but its position may be established by finding a characteristic convergence of rows of cilia. The mouth is capable of great distention during ingestion of food and during elimination of undigested material, such as diatom frustules.

The principal elements of the nervous system in this small acoel are not clear in any of my preparations. Close to the anterior end of the body, in front of the statocyst, a ring-like concentration of nerve tissue encircles the so-called "frontal organ" (the confluence of ducts of the frontal glands approaching the pore through which their secretion is discharged). However, the nerve tissue and ectocytium are so closely bound together that I have not established how many ganglia contribute to the brain mass, and I have not been able to trace any important nerves leading away from it.

Much of the anterior quarter of the body is occupied by the frontal glands and their secretion (Figs. 6, 7). In sections, the secretion is conspicuous as a pale yellowish material which is not appreciably stained by either hematoxylin or acid counterstains such as eosin, orange G, and fast green. The pore through which the secretion is discharged is circular and is located at the anterior tip of the body. The epicytium is supplied with numerous small glands (Fig. 8) within which the bundles of rhabdites are formed. The rhabdites are destroyed by the two fixatives which I used.

The subepicytial musculature (Fig. 8) consists of an outer layer of circular muscles and an inner layer of longitudinal muscles. In the parenchyma, there are scattered longitudinal and dorsoventral muscle fibers; dorsoventral muscles traversing the endocytium near the mouth are particularly conspicuous (Figs. 6, 7). The musculature associated with the copulatory organs will be described subsequently.

In the region of the mouth, the endocytium, into which food is ingested, occupies most of the body mass mesial to the ovaries and testes (Fig. 7). The endocytium extends anteriorly as far as the frontal gland cells and posteriorly as far as the anterior edge of the seminal vesicle. The ectocytial layer, which is very thin at the level of the mouth, becomes slightly more prominent anteriorly, and is very conspicuous in the posterior quarter of the body. Dorsal, lateral, and posterior to the copulatory organs, the vacuoles in the ectocytium reach a very large size (Figs. 1, 5, 6, 7).

The testis and ovary on each side of the body are closely apposed for most of their length. In the region just behind the frontal glands, the testes are lateral and dorsal to the string of small oogonia lying near the ventral epicytium (Figs. 6, 7). As the oogonia enlarge into oocytes, and therefore occupy progressively more of the body mass as they migrate posteriorly (Fig. 9), the testes become gradually restricted to a narrow zone lateral to the oocytes.

From the posterior end of each testis, a delicate duct carries sperm through the parenchyma to the seminal vesicle (Figs. 5, 10). The two sperm ducts enter the seminal vesicle at rather widely separated points on its anterolateral surfaces. The seminal vesicle (Figs. 5, 6, 7, 11) has a thin muscular wall, and in mature specimens invariably contains inactive sperm. Within the seminal vesicle, on its posteroventral side, there is a cluster of granule-filled masses which surround a small cavity continuous with the lumen of the penis. In life, the cluster resembles a group of cells (Fig. 5), but the boundaries of the individual masses are destroyed by fixation, and I have not been able to distinguish nuclei among the granules, which are refractile and are stained by hematoxylin. The penis appears to be of a type which is everted during copulation, and presumably when this takes place at least some of the granules at its tip are discharged.

The mature sperm (Fig. 3) of *P. luteola* are about 150 μ long. Behind the appreciably thickened anterior portion (slightly over one-third of the total length), the tail of the sperm narrows gradually to a very fine tip.

The vagina, supplied externally with an outer layer of longitudinal muscles and an inner layer of circular muscles, extends at first almost directly dorsally away from the genital atrium. This lower portion of the vagina has a distinct lumen (Figs. 6, 7); the lumen becomes gradually more extensive, and crescentic in outline as it is viewed from the dorsal side in living specimens (Fig. 5). The surface of the syncytial wall of the vagina next to the lumen is covered with small granules which appear to be of the same type as those associated with the tip of the penis. Some of these granules are noted within the tissue of the vagina and also within the lumen.

As the vagina arches anterodorsally over the seminal vesicle, the lumen becomes obliterated by a syncytial mass continuous with the syncytium of the rest of the vagina, and the layer of circular muscles becomes more pronounced. In life, the syncytium contains refractile crystal-like granules (Fig. 5); in specimens which have been fixed and sectioned, the granules are not preserved, and the syncytium is conspicuously vacuolated (Figs. 7, 11). Finally, the musculature disappears, and the vagina passes insensibly into a syncytium distinct from the digestive endocytium and within which the seminal bursa develops (Figs. 5, 7, 10). In some living specimens, as well as in sectioned preparations, sperm are observed in two or more spaces which may be connected or apparently separate (Fig. 7). Extending ventrally or posteroventrally from the bursa is a heavily cuticularized nozzle invested by what appears to be a fibrous tissue (Figs. 7, 10). The sperm in the seminal bursa usually exhibit considerable activity.

How the sperm reach the syncytium, within which the rather poorly-defined seminal bursa develops, is not clear. I have not been able to distinguish a continuous clear passage through the syncytium constituting that portion of the vagina which lies above the seminal receptacle. However, sperm have been noted in the lower portion of the vagina. It is possible that when insemination is effected, the sperm entering the vagina are simply forced through the syncytium to the region where the bursa is formed.

In the parenchyma around the seminal vesicle and anterior region of the vagina, there are a number of cellular elements which appear to be gland cells. The exact distribution of these glands and the pathways by which their secretion or secretions are delivered to other organs have not been worked out.

The holotype specimen, in the form of a set of serial sagittal sections, has been deposited in the United States National Museum (USNM No. 32902).

The nature of the brain of *P. luteola,* and the fact that its vagina enters the genital atrium behind the copulatory complex, indicate that it belongs in the family Otocelididae. Westblad (1948) established this family to include a single genus, *Otocelis,* which had previously been referred to the Convolutidae by most students concerned with this general group of acoels.

Westblad (1946) recognized only two species of *Otocelis: O. rubropunctata* (Schmidt) and *O. gullmarensis* Westblad. Ax (1959) pointed out that the acoel believed by Westblad to be *O. rubropunctata* is quite distinct from the worm described by Schmidt and later studied in detail by von Graff. The true *O. rubropunctata,* which has a single genital pore, is not definitely known to occur outside the Mediterranean Sea and Black Sea. Westblad's *O. "rubropunctata,"* from Scandinavian localities, has separate male and female pores. Ax proposed that it be called *O. westbladi.*

Two other acoels have rather recently been added to the genus *Octocelis. O. dichona* Marcus (1954) is distinctive in having the genital pore located at the posterior end of the body. *O. sachalinensis* Ivanov (1952) is probably more nearly similar to *O. rubropunctata* than to any other species of *Otocelis.* However, it lacks eyes, and the organization of the penis is very much like that in *P. luteola.* Certain of Ivanov's figures suggest that the tip of the penis of *O. sachalinensis* has masses of granules similar to those associated with the penis of *P. luteola,* although Ivanov did not mention any such masses in the text.

In the acoel I have described, the most distinctive feature, not shared by any of the other known species of *Otocelis,* is the peculiar nature of the anterior part of the vagina, where the lumen appears to be obliterated. It is primarily on the basis of this characteristic of *P. luteola* that I propose a new genus. I am fully aware that *O. sachalinensis* and *P. luteola* are similar in a number of respects, but the relationship of *P. luteola* to the genotype (*O. rubropunctata*) or to the other species of *Otocelis* is probably considerably more remote.

Raphidophallus actuosus gen. nov., sp. nov.

This acoel is moderately common in the small inlet of North Bay with which Argyle Lagoon communicates. Washings of *Ulva* detached from substrates of muddy sand or gravel at tide levels ranging from about −1 to +4 ft. often contain some *R. actuosus.*

When extended and gliding actively on a firm substrate (Fig. 12), the length of *R. actuosus* is equal to about four times the width. Anteriorly, the body is rounded; posteriorly, it tapers only slightly. The largest specimens, in a normal state of extension, are about 880 μ long and 220 μ wide. When the worms are in tight contact with the substrate, the ventral surface is flattened, but the thickness in the mid-dorsal region may nearly equal the width. When swimming free of the substrate, *R. actuosus* becomes almost cylindrical.

Of the three acoels described in this paper, *R. actuosus* is the most active, and it is also the most fragile. When the animal is swimming in contact with the substrate, its movements are jerky, and the posterior part of the body is often twitched back and forth, as if it were being irritated. Addition of a very little isotonic magnesium chloride may cause it to disintegrate, and when it is under slight pressure from a coverglass it is less likely to maintain its integrity than the other two species.

The statocyst is located near the end of the first one-eighth of the body. In larger specimens, its diameter is about 20 μ. The diameter of the statolith is about 14 μ. The shape of this structure is approximately hemispherical, and the convex surface is uppermost.

Viewed with reflected light, the body (except for the digestive endocytium) is whitish. The endocytium is typically brownish yellow, but it may be greenish yellow, or partly of this color. Deposits of bright white material are usually very conspicuous in the dorsal part of the body around the statocyst, and in a broken streak extending for most of the length of the body along the midline.

In strong transmitted light, the diatoms taken into the digestive endocytium are observed to change from a yellow-brown or olive color to greenish yellow and brownish yellow. The pigments diffuse out of the frustules and color the endocytium as a whole. Small lipid droplets are abundant in the ectocytial parenchyma, especially in the anterior half of the body. Most of these are yellow in color, and occur in clusters of various shapes; some clusters contain a large number of droplets, and are confluent with other clusters. The bright white deposits noted around the statocyst and along the midline when the worms are studied in reflected light are aggregates of small refractile granules which individually are pale yellowish green in color. The aggregates, however, appear blackish or brownish. They lie within the ectocytial parenchyma in the dorsal part of the body.

The body is entirely covered by cilia about 8 or 9 μ long. Scattered over the body surface are stiff, cilia-like bristles. Most of these are approximately 15 μ long, but in the caudal region some of the bristles may reach a length of nearly 30 μ.

The bundles of rhabdites of *R. actuosus* are conspicuous because they are closely spaced and are arranged in definite rows (Fig. 13). A particular row of rhabdites is never very long, however, and eventually merges with another row or terminates as two other rows converge.

The mouth is located on the ventral surface just posterior to the middle of the body. It is capable of rapid distention during the ingestion of food and elimination of undigestible residues (largely diatom frustules).

The brain appears to consist of four major ganglia, one anterolateral and one posterolateral to the statocyst on either side. Heavy commissures connect the ganglia in front of and behind the statocyst, so that the latter is almost completely enclosed within nerve tissue. My preparations do not clearly show the nerve trunks which originate from these ganglia.

The frontal glands and their accumulated secretion occupy a considerable part of the body mass in the vicinity of the statocyst (Fig. 17). The secretion appears in sectioned specimens as a pale yellowish material. The circular pore through which the secretion is discharged is located at the anterior end of the body, but is directed slightly downward. The epicytium is supplied with many small glands (Fig. 18) within which the bundles of rhabdites are formed.

The subepicytial musculature (Fig. 18) consists of an outer layer of circular muscles and an inner layer of longitudinal muscles. Parenchymal muscles are also present; these are most conspicuous in the anterior part of the body, in the region occupied by the frontal glands.

The digestive endocytium reaches anteriorly to the frontal glands and posteriorly nearly to the back edge of the seminal vesicle (Fig. 17). The ectocytial layer is rather thin in most regions of the body. It becomes appreciably thicker near the anterior end, but is most extensively developed in the posterior part of the body, where vacuoles within it are conspicuous lateral, dorsal, and posterior to the copulatory organs (Figs. 12, 15, 16, 17).

The testes and ovaries are approximately one-half the length of the body, and anteriorly they reach nearly to the level of the statocyst. In front of the mouth, the testes form a considerable part of the body mass. They are located above the string of enlarging oogonia, and their distribution is lateral and dorsal to the endocytium (Figs. 19, 20). For about one-half of their length, the testes of the right and left sides are confluent dorsally. However, as the endocytium becomes limited to the dorsal part of the body and the oocytes beneath it become very large, the testes become distinctly separate, and finally they occupy only a very small portion of the body mass as seen in transverse sections.

The genital pore (Figs. 15, 17) is located on the ventral surface near the beginning of the last quarter of the body. In living specimens, the genital atrium contains a number of more or less ovoid masses of granular material (Fig. 15). The source of these is not known. In sections of fixed specimens, the secretion within the genital atrium has the appearance of a vacuolated coagulum (Fig. 17); small granules which are stained by iron hematoxylin are scattered through this.

The seminal vesicle (Figs. 15, 16, 17, 21) is located posterodorsal to the genital atrium. The sperm ducts leading from the testes enter it at rather widely separated points on its anterolateral surface. The wall of the seminal vesicle is thin but muscular. The penis, as seen in sagittal sections (Fig. 17), arches from the genital atrium through the upper part of the seminal vesicle, and its lumen communicates with the cavity of the seminal vesicle at the rear. The penis is invested externally by an outer layer of longitudinal muscles and an inner layer of circular muscles, and its lumen is provided with a number of separate and very delicate cuticular rods. Presumably, the penis is everted during copulation. The cavity of the seminal vesicle is filled with inactive sperm. It is crescentic in outline when viewed from above, but it extends farther anteriorly near the ventral side of the seminal vesicle than near the dorsal side. Between the sperm mass and the penis there is a large accumulation of granules which are conspicuous in living specimens (Fig. 15) and which are stained strongly by hematoxylin (Figs. 16, 17, 21).

The mature sperm (Fig. 14) are about 110 μ long, and their structure is very interesting. Behind the thickened anterior region, a number of delicate cilia-like projections extend away from the sperm. The basal portions of these projections are appreciably thicker than the slightly longer outer portions. The posterior region of the sperm narrows gradually to a very fine tip.

The vagina (Fig. 17) extends anterodorsally from the genital atrium. It is short, and a part of it is ciliated. Where the lumen terminates, there are glands which produce the elongate clusters of granules often noted anterior to the genital pore in living specimens (Fig. 15). When worms of this species are compressed, the clusters of granules may actually protrude from the genital pore. In sectioned

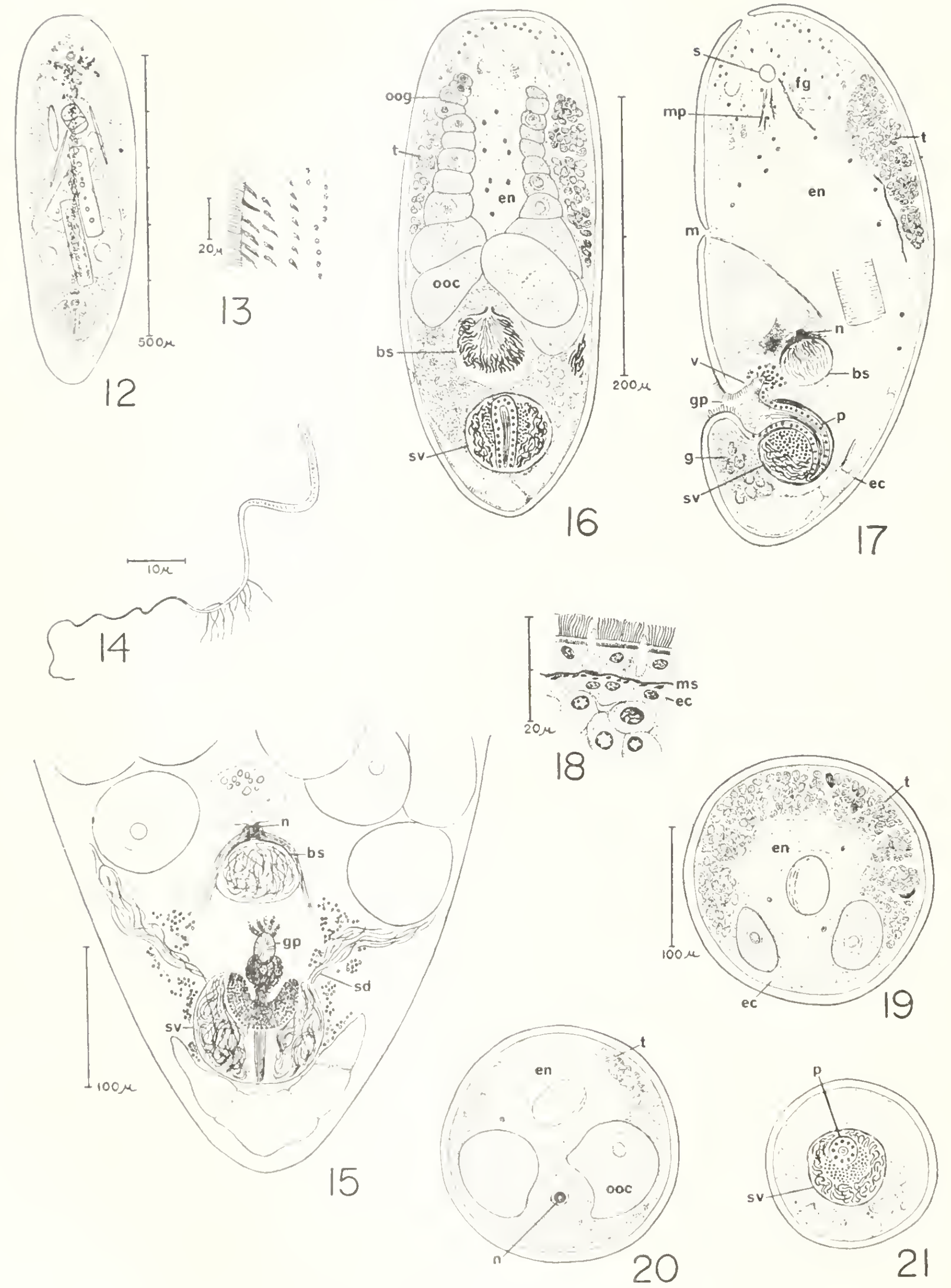
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Plate II—*Raphidophallus actuosus*

All figures were prepared with the aid of a camera lucida, but in the case of specimens drawn from life (Figs. 12–15), most details were sketched in free-hand. Figures 16–21 are based on sections (6 μ) of specimens fixed in Bouin's fluid and stained with iron hematoxylin preparations, only traces of the clusters can be detected in the anteriormost part of the wall of the vagina, and a brownish-yellow coagulum is noted in the lumen of the vagina near them. Between the glandular cap of the vagina and the seminal bursa, there is a syncytial mass within which the nuclei are rather close together, and through which sperm are probably forced into the bursa at the time of insemination.

The holotype specimen, in the form of a set of serial frontal sections, has been deposited in the United States National Museum (USNM No. 32903). It was collected in the small inlet of North Bay which communicates with Argyle Lagoon, San Juan Island, Washington.

This acoel can be referred to the family Convolutidae, and may be rather closely related to certain of the diverse species of *Convoluta*. The penis lies within the seminal vesicle in much the same manner as that of *C. divae* Marcus (1950), *C. norvegica* Westblad (1946), and *C. flavibacillum* Jensen (see Westblad, 1946), but the presence of numerous cuticularized rods within the penis is distinctive. I base the genus *Raphidophallus* largely on this combination of characters.

Diatomovora amoena gen. nov., sp. nov.

This relatively large species is usually found in washings of *Ulva* growing on muddy sand or gravel in the small inlet of North Bay which communicates with Argyle Lagoon. Samples collected at tide levels ranging from −1 to +4 ft. generally contain *D. amoena;* as a rule, it is more abundant than *R. actuosus.* I have also found it in washings of *Ulva* and *Enteromorpha* taken from muddy sand in South Slough at Charleston, Oregon (Lat. 43° 20.4′ N.; Long. 124° 19.5′ W.) at tide levels of about +2 to +5 ft.

When extended and gliding in contact with a firm substrate (Fig. 22), the body is about four times as long as wide. The largest specimens are about 1200 μ by 300 μ. The body is rounded anteriorly and tapers slightly toward the posterior end. Although the body is usually widest near the middle, the width remains almost constant in the second and third quarters. The greatest thickness, just behind the middle of the body, is nearly equal to the width. When the worms are gliding on a firm substrate, the ventral surface is flattened; when swimming free, the body becomes nearly cylindrical.

(some preparations were counterstained with orange G); certain details were supplied from adjacent sections in the same series. For abbreviations, see legend for Figures 1–11.

FIGURE 12. Specimen in contact with substrate; dorsal view.

FIGURE 13. Rhabdites near left margin of body at the level of the statocyst.

FIGURE 14. Mature sperm.

FIGURE 15. Posterior end (specimen slightly compressed under a coverglass); ventral view. The masses of granular material shown next to the genital pore lie within the genital atrium.

FIGURE 16. Frontal section. In the anterior part of the body, the section shows the region slightly ventral to the level of the statocyst and pore of the frontal glands; one of the oocytes is undergoing a maturation division.

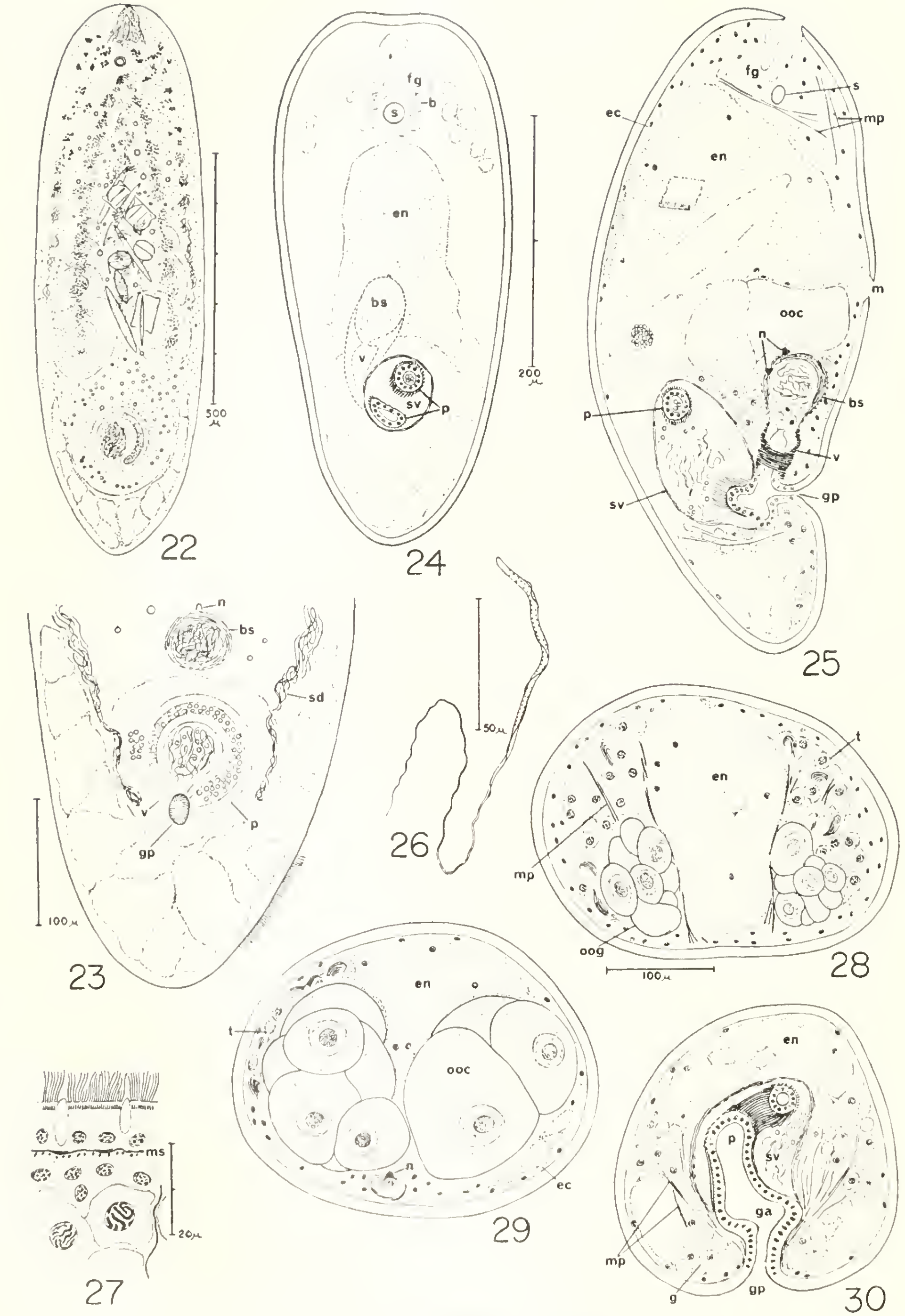
FIGURE 17. Median sagittal section.

FIGURE 18. Epicytium, epicytial glands, subepicytial musculature, and portion of testis in transverse section just anterior to mouth.

FIGURE 19. Transverse section just anterior to mouth.

FIGURE 20. Transverse section through nozzle of seminal bursa of same specimen.

FIGURE 21. Transverse section through seminal vesicle and penis of same specimen.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30

Plate III—*Diatomovora amoena*

All figures were prepared with the aid of a camera lucida, but in the case of specimens drawn from life (Figs. 22, 23, 26), most details were sketched in free-hand. Figures 24, 25, The statocyst lies near the end of the first one-tenth of the body. In larger individuals, its diameter is about 25 μ. The diameter of the hemispherical statolith is about 17 μ.

As viewed with reflected light, the body (excluding the digestive endocytium) is whitish, tinged with yellow; the yellow color, concentrated in lipid droplets, is prominent in the anterior quarter, and sometimes also near the posterior end. Near the dorsal surface, there are generally three discontinuous streaks of bright white material; these streaks originate behind the statocyst and diverge as they extend posteriorly. Some specimens have only one conspicuous streak along the midline; in others, the lateral streaks are distinct and the median streak is diffuse. The digestive endocytium is usually green, although sometimes it is greenish yellow.

In strong transmitted light, the ingested diatoms within the endocytium may be observed to turn from yellowish brown or yellowish green to a bluish green color. The endocytium as a whole is usually pale bluish green. Lipid droplets up to about 15 μ in diameter are scattered through the parenchyma, especially in the anterior quarter of the body. Some of these are quite yellow, and contribute to the yellowish cast noted in specimens examined in reflected light. The discontinuous streaks which appear white in reflected light are composed of greenish refractile granules (the larger of which are sculptured disks reaching a diameter of about 7 μ) concentrated in the ectocytium dorsal and dorsolateral to the endocytium. Under low magnification, the aggregates of these granules may appear blackish or brownish.

The body is covered by cilia about 10 μ long. The bundles of rhabdites resemble those of *R. actuosus;* they are closely spaced and are arranged in rather definite rows. Most of the rows are short and merge with other rows or simply terminate as the neighboring rows on either side converge toward one another.

The mouth is located on the ventral surface, just anterior to the middle of the body. It is capable of being considerably distended during the ingestion of food, which consists largely of diatoms and eggs of copepods, and during elimination of diatom frustules and other indigestible residues.

The nervous system has not been studied in detail. The brain appears to consist of four major ganglia and their commissures. There are two ganglia on either side of the statocyst—one anterolateral and the other posterolateral. These

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27–30 are based on sections (6 μ) of specimens fixed in Bouin's fluid and stained with iron hematoxylin and orange G (or eosin); in most cases, some details have been supplied from adjacent sections in the same series. For abbreviations, see legend for Figures 1–11.

FIGURE 22. Mature specimen (slightly compressed under a coverglass); dorsal view.

FIGURE 23. Posterior end (specimen slightly compressed under a coverglass); ventral view. The globules on the topographic right side of the penis are within the lumen of the vagina.

FIGURE 24. Frontal section. Most details have been omitted, and the position of the vagina and seminal bursa, which lie some distance below the level of the structures traced, are rendered diagrammatically.

FIGURE 25. Median sagittal section. The vagina and seminal bursa were drawn from a number of sections considerably to the left of the section traced.

FIGURE 26. Mature sperm.

FIGURE 27. Epicytium, epicytial glands, subepicytial musculature, and portion of testis in transverse section just anterior to mouth.

FIGURE 28. Transverse section just anterior to mouth.

FIGURE 29. Transverse section through anterior nozzle of seminal bursa of same specimen.

FIGURE 30. Transverse section through region of genital pore and genital atrium of same specimen.

are fused almost completely, and communicate with their counterparts on the other side by thick commissures.

The frontal glands and their secretion (Figs. 24, 25) occupy much of the anterior fifth of the body. The pore through which the secretion is discharged is located at the anterior end of the body and is directed slightly downward. The glands within which the bundles of rhabdites are formed may be recognized within the epicytium (Fig. 27).

The subepicytial musculature (Fig. 27) consists of an outer layer of circular muscles and an inner layer of longitudinal muscles. Parenchymal muscles are also present. These are rather abundant in the region of the frontal glands (Fig. 25) and in the region of the copulatory organs (Fig. 30). However, parenchymal muscles having a nearly dorsoventral orientation are also prominent lateral to the endocytium near the mouth (Fig. 28).

In the region of the mouth, much of the body mass mesial to the testes and ovaries is occupied by the endocytial parenchyma into which food is ingested. The endocytium extends forward to the frontal glands (Fig. 25). Posteriorly it reaches beyond the anterior margin of the seminal vesicle, although it gradually becomes restricted to the dorsal part of the body mass, above the enlarging oocytes, seminal bursa, and seminal vesicle. The ectocytial parenchyma is rather thin where the endocytium is most extensive. It becomes appreciably better developed near the anterior end, and is very prominent in the posterior part of the body. Dorsal, lateral, and posterior to the copulatory organs, the ectocytium is characterized by large vacuoles (Figs. 22, 23, 24, 25, 30).

The testes are distinctly separate. In the region just behind the frontal glands, the testis on either side of the endocytium occupies nearly a third of the body mass. Farther posteriorly, however, the oogonia which are ventral to each testis become progressively larger, and the testes become gradually more restricted (Figs. 28, 29).

The genital pore (Figs. 23, 25, 30) is located on the ventral surface near the beginning of the last one-sixth of the body. The genital atrium has a ciliated epithelium and is externally lightly muscularized. The penis has a heavy external musculature consisting of an outer layer of longitudinal fibers and an inner layer of circular fibers. Its lumen is filled with globules which consist of small granules. In some preparations, these granules are stained distinctly by hematoxylin; as a rule, however, hematoxylin seems to be removed from them by routine destaining, and they become readily colored by eosin and orange G.

The penis leaves the left side of the genital atrium and follows a nearly circular ascending path through the seminal vesicle. The cavity of the seminal vesicle is filled with inactive sperm and globules of the type observed within the lumen of the penis. The delicate sperm ducts passing posteromedially from the testes enter the seminal vesicle on its dorsolateral surfaces.

The mature sperm (Fig. 26) are approximately 250 μ long. From the thickened anterior portion, the body becomes gradually narrowed to a very fine tip. A conspicuous undulating membrane spirals around the anterior two-thirds of the sperm. Undulations of the more slender posterior region appear to be continuous with those of the undulating membrane, and perhaps the latter extends almost the entire length of the sperm.

The vagina (Figs. 24, 25) has a thick tunic of circular muscles, and leaves the genital atrium ventral to the seminal vesicle. It is directed at first slightly to the right, then bends toward the midline on its course to the seminal bursa. For most of the length of the vagina, the lumen is distinct, and this may contain a number of globules of the type noted within the seminal vesicle and penis. Just before the vagina reaches the bursa, the lumen is obliterated by a syncytial mass continuous with the syncytial wall of the vagina. In life, this mass often contains clusters of refractile granules, and is very similar to that which obliterates the lumen of the anterior part of the vagina in *P. luteola*. In fixed and sectioned specimens, it is vacuolated and the granules are not preserved. The seminal bursa is invested by a heavy coat of fibrous elements; external to this, there are some widely-spaced muscles.

The bursa has two cuticularized nozzles (Fig. 25); one of these is directed almost anteriorly, and the other is usually directed dorsally or anterodorsally. The bursa generally contains active sperm, but in some specimens it is filled with the granule-bearing syncytium which usually characterizes the anterior portion of the vagina. It appears likely that unless the bursa is distended by sperm forced into it at the time of insemination, it tends to collapse and thus appears to envelop the syncytial mass just behind it.

Gland cells are extensively developed in the parenchyma around the copulatory organs, but I have not worked out the pathways by which the secretion or secretions of these glands are delivered to other structures.

The holotype specimen, in the form of a set of serial sagittal sections, has been deposited in the United States National Museum (USNM No. 32904). It was collected in the inlet of North Bay which communicates with Argyle Lagoon, San Juan Island, Washington.

Like the preceding species, this acoel belongs in the Convolutidae. Its long and highly muscular penis is somewhat similar to that of *Aphanostoma macrospiriferum* Westblad (1946) and *A. rhomboides* (Jensen) (see Westblad, 1946). However, neither of these species has a seminal bursa. When a bursa is present in members of the genus *Aphanostoma,* it does not have a cuticularized nozzle, although Westblad has questioned the importance of this characteristic in setting *Aphanostoma* apart from *Convoluta*. The heavily muscularized vagina of *D. amoena* and the nature of its seminal bursa, which has two nozzles and a thick wall consisting of fibrous elements surrounded by muscles, are characteristics which have persuaded me to propose a new genus.

Summary

Acoels belonging to three new genera are described. *Parotocelis luteola* is referred to the family Otocelididae. *Raphidophallus actuosus* and *Diatomovora amoena* are placed in the family Convolutidae. All of these acoels have been collected intertidally on San Juan Island, Washington, on substrates of muddy sand and gravel supporting growths of *Ulva* and *Enteromorpha*. *D. amoena* has also been found at Charleston, Coos County, Oregon.

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THE SEPARATION OF POST-BASICORONAL AREAS FROM THE BASICORONAL PLATES IN THE INTERAMBULACRA OF THE SAND DOLLAR, ECHINARACHNIUS PARMA (LAMARCK)[1]

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One possible arrangement of the coronal plates of the central portion of the oral surface of the test of the sand dollar *Echinarachnius parma* (Lamarck) is shown as Figure 1. In this specimen the post-basicoronal interambulacral areas one through four are in contact with the basicoronal plates, but in interambulacrum five the post-basicoronal area has become separated from its basicoronal plate. Durham (1955) has indicated that the geologically younger genera of scutellinid echinoids tend to have the interambulacral columns separated from the basicoronal plates, whereas in the older genera these columns and plates are in contact. He also noted that in the Pacific Coast sand dollar, *Dendraster excentricus* (Eschscholtz), a member of one of the younger or more advanced genera, very small or young individuals had their basicoronal interambulacral plates in full contact with the succeeding plates and that as growth proceeds, the second plate of each ambulacral column grows faster than the others and eventually separates the second interambulacral plate from contact with the basicoronal interambulacral plate. Of all the species he studied for variation, *Echinarachnius parma* was found to be the most variable in respect to the separation of the interambulacral columns from the basicoronal plates. This study has been made with the aim of determining whether any pattern can be noted in this variation. With this in mind three questions are posed:

1. How many areas lose contact, and to what extent does this vary among specimens within and between collections from different localities?
2. Is there indication that there is any usual sequence among the areas in their loss of contact, and does this vary within and between collections from different localities?
3. Within areas retaining contact, are there differences in the amount of contact between first post-basicoronal plates "a" and "b" with the basicoronal plates? Is there any regular pattern of distribution of this asymmetry among the areas, and does this vary within and between collections from different localities?

MATERIALS AND METHODS

Four series of specimens were collected intertidally, one series from each of the following places: Crow Neck, North Trescott, Washington County, Maine (44° 52′ 37″ N., 67° 07′ 38″ W.); Bailey's Mistake, South Lubec, Washington

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[1] The greater part of this work was included in a dissertation submitted by the senior author to the University of New Hampshire in partial fulfillment of the requirements for the Ph.D. degree.

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County, Maine (44° 46′ 23″ N., 67° 03′ 16″ W.); Hampton Beach, Rockingham County, New Hampshire (42° 54′ 07″ N., 70° 48′ 40″ W.); and Hampton Harbor, Rockingham County, New Hampshire (42° 53′ 59″ N., 70° 49′ 07″ W.). To minimize the possibility of the introduction of variability resulting from possible seasonal differences these collections were all made within as short a time period as feasible (September 12–15, 1962). Pertinent environmental characteristics of these collecting localities have been discussed by Lohavanijaya (1965).

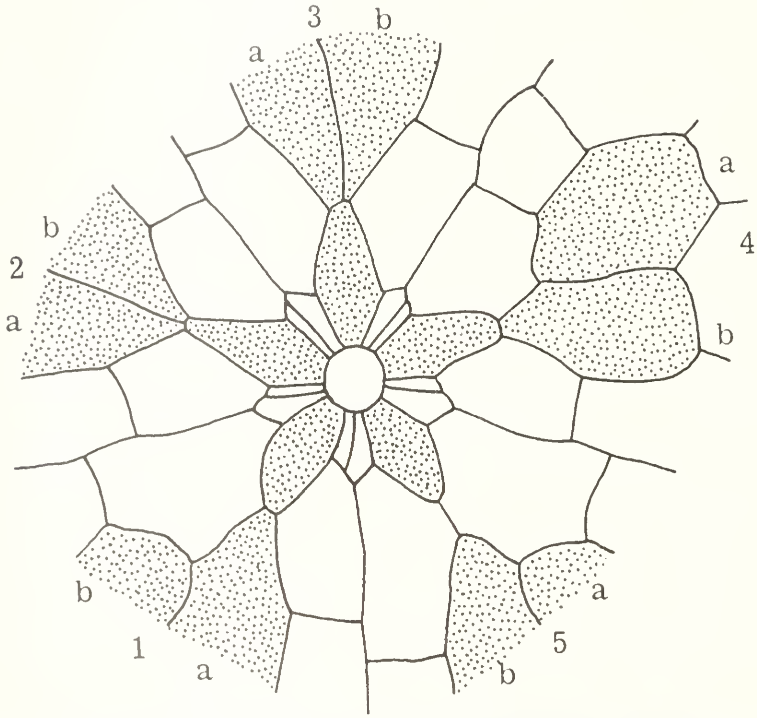

FIGURE 1. Oral surface of central portion of test of *Echinarachnius parma,* showing contact or lack of contact between basicoronal interambulacral plates and first post-basicoronal plates. Stippled areas are interambulacral and white areas are ambulacral.

More careful examination of the specimen shown in Figure 1 reveals not only that the post-basicoronal interambulacral areas 1, 2, 3, and 4 are in contact with their basicoronal plates, but also that the nature of this contact varies. In area 1 the first post-basicoronal plate "a" is in contact but "b" has lost contact, whereas in area 4 the situation is reversed. In areas 2 and 3 both "a" and "b" remain in contact, but it looks as though "b" were approaching loss of contact in area 3 while in area 2 the degree of contact appears more nearly equal.

In order to tabulate such variants for the large numbers of specimens studied, the following system of symbols has been devised.

If both plates "a" and "b" of the first post-basicoronal interambulacrals are "in contact" with the basicoronal to an approximately equal degree, the condition is designated: + +

If both plates "a" and "b" are "in contact" but "a" is to a greater degree, the condition is designated: + −

If both plates "a" and "b" are "in contact" but "b" is to a greater degree, the condition is designated: − +
If only plate "a" is "in contact," the condition is designated: + ○
If only plate "b" is "in contact," the condition is designated: ○ +
If both plates "a" and "b" are "out of contact," the condition is designated: ○ ○

Such data were compiled for the five interambulacral areas for a total of 1280 specimens. There were a few specimens for which these relationships could not be determined, a few that were malformed, injured, or otherwise so abnormal that they were not considered typical, and a very few so far from the usual sizes within each series that they were considered unusual. The specimens (23) in these categories have not been included in this study. The spines and the superficial organic material on the oral surface of the test were brushed off thoroughly. Then, in order to make the sutures separating the plates more readily visible, water was applied to the test. For determination of the nature of the contact between the basicoronal plates and the post-basicoronal areas, examination of the specimens

TABLE I

The nature of contact between first post-basicoronal interambulacral plates and basicoronal plates for the five areas of the oral surface of the test for series of specimens from four localities. N = the total numbers of specimens in each series. The numbers in the bulk of the table represent the numbers of specimens having each possible type of contact or absence of it (00) for each interambulacral area

Area	a b	a b	a b	a b	a b	a b	N
	+ +	+ −	+ 0	0 0	0 +	− +	
BM* 1	2	56	72	161	1	1	293
2	32	19	5	85	36	116	
3	52	94	26	89	11	21	
4	2	0	0	116	98	77	
5	7	2	2	210	14	58	
CN* 1	8	73	106	105	2	1	295
2	30	17	5	68	79	96	
3	61	71	39	53	19	52	
4	2	3	1	88	109	92	
5	8	0	0	225	28	34	
HH* 1	11	149	76	68	0	7	311
2	50	67	7	60	22	105	
3	72	115	21	46	2	55	
4	7	3	0	65	70	166	
5	34	10	2	89	41	135	
HB* 1	39	171	68	44	37	11	370
2	122	58	6	31	19	134	
3	151	89	10	11	15	94	
4	39	18	1	58	56	198	
5	88	9	0	62	57	154	

* BM—Bailey's Mistake; CN—Crow Neck; HH—Hampton Harbor; HB—Hampton Beach.

TABLE II

Comparison of frequency of occurrence of specimens with the "normal," "1st order" and "2nd order" deviant arrangements of interambulacral areas "out of contact" for specimens with 0, 1, 2, 3, 4 or all areas "out of contact" for Echinarachnius parma from the four localities studied

Series	# of specimens	% of collection	# of areas "out of contact"	Normal sequence		Deviant sequences			
						1st order**		2nd order**	
				#	%*	#	%	#	%
CN (15–70 mm.)	74	24.7	0	74	100.0	Not possible		Not possible	
	93	31.1	1	91	97.8	2	2.2	Not possible	
	36	12.0	2	18	50.0	18	50.0	0	0.0
	41	13.7	3	20	48.8	15	36.6	6	14.6
	34	11.4	4	13	38.2	15	44.1	6	17.6
	21	7.0	all	21	100.0	Not possible		Not possible	
Average number of areas "out of contact" per specimen = 529/299 = 1.77									
BM (50–90 mm.)	68	22.4	0	68	100.0	Not possible		Not possible	
	48	15.8	1	39	81.3	9	18.8	Not possible	
	45	14.9	2	29	64.4	14	31.1	2	4.4
	58	19.1	3	29	50.0	19	32.8	10	17.2
	40	13.2	4	18	45.0	15	37.5	7	17.5
	44	14.5	all	44	100.0	Not possible		Not possible	
Average number of areas "out of contact" per specimen = 692/303 = 2.28									
HB (20–59 mm.)	241	65.1	0	241	100.0	Not possible		Not possible	
	56	15.1	1	33	58.9	23	41.1	Not possible	
	21	5.7	2	9	42.9	4	19.0	8	38.1
	26	7.0	3	19	73.1	4	15.4	3	11.5
	14	3.8	4	11	78.6	3	21.4	0	0.0
	12	3.2	all	12	100.0	Not possible		Not possible	
Average number of areas "out of contact" per specimen = 292/370 = 0.79									
HH (40–65 mm.)	182	58.7	0	182	100.0	Not possible		Not possible	
	44	14.2	1	24	54.5	20	45.5	Not possible	
	26	8.4	2	2	7.7	12	46.2	12	46.2
	18	5.8	3	8	44.4	2	11.1	8	44.4
	22	7.1	4	11	50.0	4	18.2	7	31.8
	18	5.8	all	18	100.0	Not possible		Not possible	
Average number of areas "out of contact" per specimen = 328/310 = 1.06									

* These percentages refer to the percentage of specimens with the indicated number of areas "out of contact" having the sequence indicated.

** First order deviants are those presumed to be normal except for last one out of contact. Second order deviants are those combinations in which there appears to be aberrance in sequence in loss of contact prior to the last area involved.

with a hand lens was necessary. Each series of specimens was divided into size groups at 5-mm. intervals (except where numbers were inadequate). The data thus obtained were tabulated. In Table I these data are summarized by locality and area of test but without breakdown into size groups.

TABLE III

Distribution of numbers of interambulacral areas "out of contact" among different size groups of Echinarachnius parma from four localities

Series	Mean diameter ½ (L + W)	No. of specimens	Number of areas "out of contact"						Mean
			0	1	2	3	4	5	
CN	15–23.9	10	8	1	1	—	—	—	0.30
	27–34.9	7	4	1	1	1	—	—	0.86
	35–39.9	15	5	4	2	3	—	1	1.47
	40–44.9	38	11	12	2	5	6	2	1.71
	45–49.9	42	8	10	6	10	5	3	2.07
	50–54.9	63	12	22	11	7	8	3	1.78
	55–59.9	67	11	24	10	7	10	5	1.94
	60–64.9	38	11	13	2	5	4	4	1.76
	65–69.9	19	4	7	1	3	1	3	1.95
BM	50–54.9	30	7	9	3	4	6	1	1.87
	55–59.9	66	17	9	14	7	11	8	2.15
	60–64.9	59	21	8	4	13	5	8	1.95
	65–69.9	52	5	11	7	11	7	11	2.71
	70–74.9	46	6	4	11	11	4	10	2.72
	75–79.9	40	12	5	5	10	3	5	2.05
	80–84.9	10	—	2	1	2	4	1	3.10
HB	20–24.9	19	18	1	—	—	—	—	0.05
	25–29.9	24	22	2	—	—	—	—	0.09
	30–34.9	51	39	6	3	1	—	2	0.49
	35–39.9	56	37	11	3	2	1	2	0.66
	40–44.9	91	59	12	4	9	5	2	0.85
	45–49.9	81	46	16	5	8	3	3	0.95
	50–54.9	38	16	7	4	6	2	3	1.47
	55–59.9	10	4	1	2	—	3	—	1.70
HH	40–44.9	15	9	2	—	1	2	1	1.20
	45–49.9	75	44	12	7	4	5	3	0.97
	50–54.9	121	71	19	8	7	7	9	1.07
	55–59.9	83	54	7	9	4	4	5	0.94
	60–64.9	16	4	4	2	2	4	—	1.88

NUMBERS OF AREAS OUT OF CONTACT

In Table II several kinds of data are tabulated. The first column on the left indicates the number of specimens from the locality indicated having 0, 1, 2, 3, 4, or all areas "out of contact." In the next column these numbers have been converted into percentages of the total number of specimens used in this study from each locality. Then under the tabulations for each locality the average number of areas "out of contact" per specimen for the collection from the locality has been calculated. The average numbers of 1.77 for Crow Neck, 2.28 for Bailey's Mistake, 0.79 for Hampton Beach, and 1.06 for Hampton Harbor suggest that the Maine localities have populations that are more progressive in this respect than are those from the New Hampshire sites. Noting the size ranges from the localities (indicated on the table under the initials for the name of each locality) and recalling that numbers of areas "out of contact" presumably increase as the animals grow, one is immediately beset with the question: Are these differences the result of differences in environmental induction or selection on the one hand, or are they wholly the result of the differences in size-composition among the collections? Table III and Figure 2 have been assembled to show the mean numbers of areas "out of contact" at 5-mm. size (mean diameter) intervals for each of the localities. It is obvious that much larger collections and smaller size intervals would be needed to give smooth curves on the graphs, but it is quite apparent that:

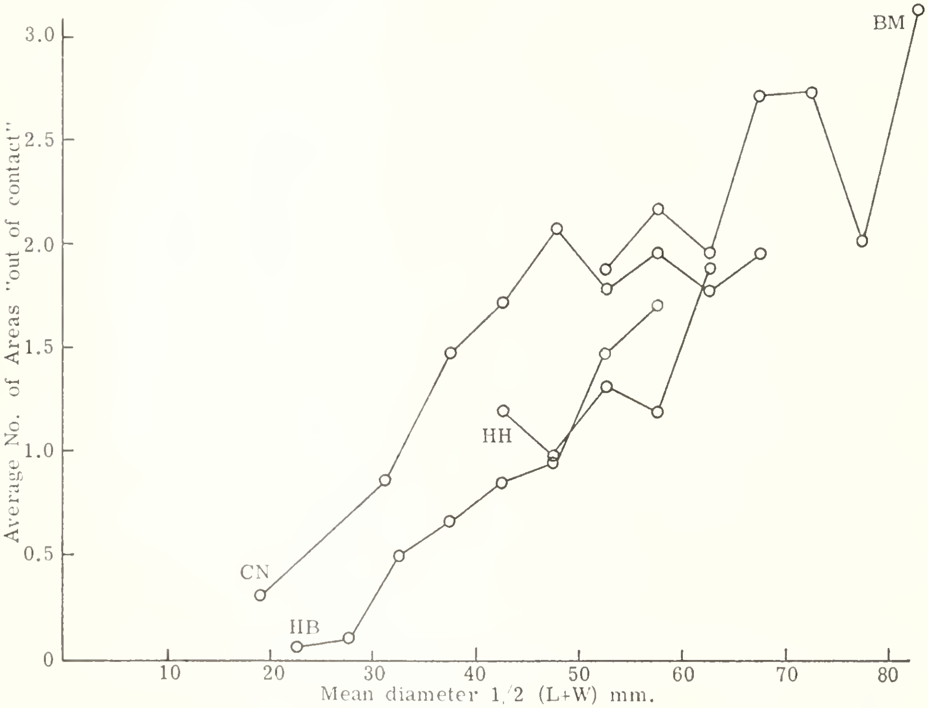

FIGURE 2. Relationship of the average number of areas "out of contact" to size ½ (L+W).

(1) For comparable mean diameters up to at least 55 mm. the mean number of areas "out of contact" is higher for the Crow Neck collection than for either Hampton Beach or Hampton Harbor, and

(2) In the range of diameters between 55 and 70 mm. there appears possibly to be a tendency toward equal numbers of areas "out of contact" for all the localities. Thus for mean diameters of 62.5 mm., the mean numbers of areas "out of contact" for the collections from Crow Neck, Bailey's Mistake, and Hampton Harbor all

fall within the range between 1.75 and 2.00. Although none of the specimens from Hampton Beach is this large, extrapolation of the plotted values for smaller sizes into this range would place the expected value for this locality very close to 2.00 areas "out of contact."

Apparent Sequence of Loss of Contact in Interambulacral Areas

All possible combinations of areas "out of contact" were listed, and for each locality the number of specimens having each combination was tallied. Examination of these data along with Durham's (1955) Table 3 (p. 108) strongly suggested that the usual sequence in which interambulacral areas lose contact is 5, 1, 4, 2, 3. Thus, one would expect specimens "out of contact" for a single area to be most frequently "out of contact" in area 5. When two areas are "out of contact," areas 5 and 1 should be the most frequent combination. This would continue, and the whole expected sequence would thus be 0→5→5 & 1→5, 1, & 4→5, 1, 4, & 2→5, 1, 4, 2, & 3 (= all areas). These combinations are hereafter called members of the normal sequence. Durham's (1955) data for his series from Woods Hole, Massachusetts, and the data here presented in Table II for Crow Neck, Bailey's Mistake, and Hampton Beach support this sequence, or at least the resulting combinations that may be obtained through it. Thus, these combinations of areas "out of contact" are the most frequently occurring combinations in the collections aforementioned. Durham (1955) noted that the small collection (21 specimens) he studied from Hampton Harbor exhibited great variation in respect to loss of contact among the interambulacral areas. In the present study 310 specimens were examined from this locale, and Durham's conclusion is abundantly supported as can readily be seen from examination of Table II.

TABLE IV

All possible combinations of normal, 1st order deviants, and 2nd order deviants

Areas "out of contact"	Normal	1st order deviants	2nd order deviants
0	All	Not possible	Not possible
1	5	1; 2; 3; or 4	Not possible
2	5 & 1	5, 2; 5,3; or 5,4	1, 2; 1, 3; 1, 4; 2, 3; 2, 4; or 3, 4
3	5, 1 & 4	5, 1, 2; or 5, 1, 3	1, 2, 3; 1, 2, 4; 1, 3, 4; 2, 3, 4; 2, 3, 5; 2, 4, 5; or 3, 4, 5
4	5, 1, 4 & 2	5, 1, 4 & 3	1, 2, 3, 4; 1, 2, 3, 5; or 2, 3, 4, 5
5 (all)	All	Not possible	Not possible

The variants from these usual combinations may be conveniently divided into two categories. Cases where combinations include areas "out of contact" in the normal combination, except for the area presumed last to lose contact, are termed "first order deviants." Thus, any specimen having one area "out of contact" other than area 5 would be a "first order deviant." "First order deviants" with two areas "out of contact" must have one of these areas 5, and the other must not be area 1. "Second order deviants" are those combinations which do not include the presumed penultimate area among those "out of contact." Thus, for specimens with two areas "out of contact," a "second order deviant" must not include area 5 among the areas "out of contact." In Table IV all possible combinations of "normal," "first order deviants," and "second order deviants" are indicated. All the theoretically possible combinations have actually been observed among the 1282 specimens dealt with in this section, except 2, 3 and 4.

Tables V and VI indicate the numbers of each particular deviant found in each collection. In Table II the occurrence of "normal," "first order deviant," and "second order deviant" combinations are totalled for each collection for each number of areas "out of contact."

TABLE V

Number of specimens of 1st order deviants in each collection

Areas "out of contact"	1st order deviants	CN	BM	HB	HH	Total
0	Not possible					
1	1	0	3	15	7	
	2	0	3	1	6	
	3	1	3	1	5	
	4	1	0	6	2	
		2	9	23	20	54
2	5 & 2	11	6	0	4	
	5 & 3	2	2	1	2	
	5 & 4	5	6	3	6	
		18	14	4	12	48
3	5, 1 & 2	7	6	3	2	
	5, 1 & 3	8	13	1	0	
		15	19	4	2	40
4	5, 1, 4 & 3	15	15	3	4	37
5 (all)	Not possible					
						179

Examination of these tables reveals that for the collections from Crow Neck and Bailey's Mistake, Maine, and Hampton Beach, New Hampshire, it is almost a generalization that for each number of areas "out of contact" there are more specimens with "normal" arrangements than with either first or second order deviant arrangements. The unique exception to this statement occurs among the specimens from Crow Neck with four areas "out of contact." Among these 38.2% have the "normal" arrangement and 44.1% have the only possible first order deviant arrangement—that is, areas 5, 1, 4, and 3 "out of contact." There are, however, a few other situations (numbers of areas "out of contact" for given localities) where the sum of the first and second order deviants exceeds the number of "normal" individuals. But for the exception noted above, however, in no case does the number of individuals with any specific deviant even approach the number of "normal" individuals in these localities.

For the collection from Hampton Harbor the situation is quite different. Although there is still a majority of these specimens with one area "out of contact" having the normal area 5 "out of contact," the percentage of these is much lower than found for the Maine localities and somewhat less than at Hampton Beach. In the group with two areas "out of contact" there is a total of only 26 specimens.

Table VI

Number of specimens of 2nd order deviants in each collection

Areas "out of contact"	2nd order deviants	CN	BM	HB	HH	Total
0	Not possible					
1	Not possible					
2	1 & 2	0	0	1	1	
	1 & 3	0	0	0	2	
	1 & 4	0	2	5	3	
	2 & 3	0	0	1	2	
	2 & 4	0	0	1	3	
	3 & 4	0	0	0	1	
		—	—	—	—	
		0	2	8	12	22
3	1, 2 & 3	0	1	0	1	
	1, 2 & 4	0	2	1	1	
	1, 3 & 4	0	1	0	1	
	2, 3 & 4	0	0	0	0	
	2, 3 & 5	3	4	0	3	
	2, 4 & 5	2	0	2	2	
	3, 4 & 5	1	2	0	0	
		—	—	—	—	
		6	10	3	8	27
4	1, 2, 3 & 4	1	0	0	4	
	1, 2, 3 & 5	1	5	0	2	
	2, 3, 4 & 5	4	2	0	1	
		—	—	—	—	
		6	7	0	7	20
5 (all)	Not possible					—
						69

Of these only two (7.7%) have the "normal" arrangement. While not much significance can be attached to these small numbers, it is interesting to note that the first order deviant arrangements of areas 5 and 4, and 5 and 2 and the second order deviants 1 and 4, and 2 and 4 "out of contact" all exceed the "normal" arrangement. These conditions suggest a tendency for areas 4 and 2 to lose contact ahead of sequence. Durham's (1955) data for this locality also indicate the tendency for area 4 to precede area 1. Second order deviants for specimens with three or four areas "out of contact" are also exceptionally high in this collection.

Here again we suffer from small numbers, but the tendency could be readily explained on the basis of deviant sequences early in their development.

Why the sequence of loss of contact among the interambulacral areas is so unusual at Hampton Harbor is a difficult question to approach. It seems incomprehensible that the Hampton Harbor population is genetically isolated from those of Hampton Beach hardly a mile distant by interconnecting water. However, there still exists the possibility that even from a common gene pool and common reservoir of larvae, there could be a selective difference of survival among genotypes after metamorphosis. The other likely explanation is that the differences in environmental factors between these proximate localities may affect genetically-like organisms in such manner that they develop differently. Regardless of whether these differences are genetic or environmentally induced, there still remains the question as to what environmental factors might be involved. A somewhat similar problem concerning variation in the heart-urchin, *Echinocardium cordatum* Pennant, as it occurs in British and nearby waters has been carefully studied by Nichols (1962), who suggests functional advantages for the variants he studied and favors the explanation of differences between populations as resulting from differential selection.

TABLE VII

Numbers and percentages of specimens, asymmetrical around the interambulacral radii, having more contact with "a" than "b" (+− and +0) and having more contact with "b" than "a" (0+ and −+) according to area of test and locality of collection

Area	Series	+ − and + 0		0 + and − +	
		No.	%	No.	%
1	CN	179	98.4	3	1.6
	BM	128	98.5	2	1.5
	HB	239	83.3	48	16.7
	HH	225	97.0	7	3.0
2	CN	22	11.2	175	88.8
	BM	24	13.6	152	86.4
	HB	64	29.5	153	70.5
	HH	74	36.8	127	63.2
3	CN	110	60.8	71	39.2
	BM	120	78.9	32	21.1
	HB	99	47.6	109	52.4
	HH	136	70.5	57	29.5
4	CN	4	2.0	201	98.0
	BM	0	0.0	175	100.0
	HB	19	7.0	254	93.0
	HH	3	1.3	236	98.7
5	CN	0	0.0	62	100.0
	BM	4	5.3	72	94.7
	HB	9	4.1	211	95.9
	HH	12	6.4	176	93.6

ASYMMETRY WITHIN INTERAMBULACRAL AREAS

Differences in the amount of contact between first post-basicoronal plates "a" and "b" with the basicoronal plates cause deviations from the symmetrical arrangements of plates on the two sides of the radius running through the middle of the area in question.

Table I summarizes the number of individuals having the various types of contact, or lack of it, between the first post-basicoronal plates and the basicoronal plates for each of the interambulacra in the specimens collected from Bailey's Mistake and Crow Neck, Maine, and Hampton Beach and Hampton Harbor, New Hampshire. Inspection of this table indicates that in areas 1, 2, 3, 4, and 5, plates "b", "a", "b", "a", and "a", respectively, appear to lose contact more frequently ahead of the other member of the pair. It can readily be seen from Table VII that the degree of preponderance varies among the areas and within areas among the collections from different localities.

DISCUSSION

The loss of contact between first post-basicoronal interambulacral plates and the basicoronal plates varies in respect to number of areas involved, apparent sequence among areas, and in the asymmetry of contact within the areas which appear to be in the process of losing contact. The number of areas "out of contact" is subject to increase as the individual grows—at least initially. Thus, specimens with (or populations averaging) more areas "out of contact" may be thought of as being more advanced or progressive. This agrees with Durham's (1955) idea that primitive genera and species near the ancestral stock retain contact whereas more highly evolved taxa are characterized by increasing loss of contact. Among the regular echinoids Jackson (1912) on similar grounds considered the exsert condition of ocular plates to be primitive and the insert condition more progressive. From the studies of Jackson (1912), Vasseur (1952), and Swan (1958, 1962) it appears that for *Strongylocentrotus* higher salinities and lower temperatures go hand in hand with the more progressive development characterized by more ocular plates insert. For the tropical *Tripneustes,* however, Jackson's (1914) data suggest the opposite relationship with temperature. *E. parma* is essentially a boreal species, and the higher numbers of areas "out of contact" in the collections from Maine, indicating that they are more progressive, might suggest that this species, like *Strongylocentrotus,* attains a more progressive condition in cooler water. Much caution should be used, however, in making even tentative conclusions on the basis of these few data. One cannot determine a trend from two points (the New Hampshire series as compared with those from Maine); and when the mean number of areas "out of contact" is calculated for Durham's (1955, Table 3, p. 108) series of *E. parma* from Woods Hole, Massachusetts, the value obtained is 1.71. At first glance it is apparent that this figure is nearly up to the overall average value for Crow Neck, Maine; but when the effect of the size of the specimens is considered, conclusions based on comparison of these overall averages become obviously questionable. If the specimens Durham (1955) used for his Table 3 are the same ones used for Table 2, which were said to range from 50 to 62 mm. in average diameter, they are in the size range where a convergence in numbers of areas "out of contact" occurs among the collections from Maine and New Hampshire and thus indicate little.

This convergence, as shown in Figure 2, makes one wonder if the loss of contact by additional areas may not cease when a certain size or age is reached. As shown by Jackson (1912) and verified by Swan (1958), this appears to be the case for ocular plates becoming insert in *Strongylocentrotus droebachiensis.* The arrangement of points (Fig. 2) relating average numbers of areas "out of contact" to mean diameter for the sand dollars from Crow Neck certainly appears to suggest a curve becoming asymptotic to the base line at some value between 1.75 and 2.00 areas "out of contact" for diameters above about 45 mm. For diameters of 45 mm. and less the "curve" for the population from Hampton Beach appears to be roughly parallel to that for Crow Neck but is displaced toward lower numbers of areas "out of contact" at comparable diameters. There is no indication of flattening out of this curve at mean diameters near 45 mm., and no specimens were available for sizes that were appreciably above the diameters where the mean number of areas "out of contact" reached 1.70. The size ranges of the series from Bailey's Mistake and from Hampton Harbor are such that they give little help toward answering questions, but the great fluctuation shown in the series from Bailey's Mistake in regard to numbers of areas "out of contact" intensifies another question suggested by the "curve" representing the Crow Neck population. If there is a limit beyond which no further areas lose contact, does the value of this limit fluctuate? If so, why? These remain as problems for future attack. Before leaving this subject, we should be reminded of the fact that Durham's (1955) findings would suggest that in sand dollars new plates on the oral side of the test are added up to a certain small size, after which no more are added. The variation he notes in the numbers of these plates in *E. parma* might be related to differences in the time at which their addition ceases in different individuals. That the addition of coronal plates may cease before regular urchins die or cease growing is indicated by Hsia (1948) for two species of *Temnopleurus.* No work is known to the present authors which indicates whether or not the size or number of plates at which this occurs varies within the species from one population to another. Again the temptation to make comparisons with better known organisms in other phyla is strong. A great many studies have been made on the numbers of vertebrae, fin-rays, and other serially repeated structures in fishes; and generally it appears that longer developmental periods (*i.e.*, slower growth through the critical stages in development) produce higher counts in meristic structures. Low temperatures, high salinities and low oxygen tensions have been shown to retard development and produce this effect. Much of the pertinent literature on this subject has been discussed and listed by Barlow (1961). That light may also affect the number of vertebrae formed appears to be the case in at least some instances (McHugh, 1954). Perhaps it is no mere coincidence that *Strongylocentrotus* appears to progress further in its attainment of insert ocular plates in colder or more saline waters and that *Echinarachnius* tends to progress toward having more interambulacral areas "out of contact" in eastern Maine than in New Hampshire. It would be interesting to check the numbers of plates on the oral surfaces of the series from colder and warmer water to see if those from colder water had a higher average number.

The sequence of 5, 1, 4, 2, 3 in which interambulacral areas lose contact is of more than a little interest. Although in *Strongylocentrotus* the normal sequence in which oculars become insert is I, V, IV, II with no record of all oculars insert, there are many genera of regular urchins where the normal sequence is V, I, IV, II, III (Jackson, 1912). Jackson (1912, 1914), Vasseur (1952), and Swan (1962) have all noted that localities differ from one another in respect to the frequency with which aberrant variant combinations of oculars insert occur in various species of regular urchins. Thus, the fact that one of the localities here studied (Hampton Harbor) is characterized by so many deviant arrangements of areas "out of contact" among its sand dollars is not surprising, but at present no explanation can be suggested. Swan (1962) has noted that certain aberrant variant arrangements of ocular plates insert in *Strongylocentrotus* are indicative of *"situs inversus."* The possibility that some of the deviant arrangements of areas "out of contact" in *Echinarachnius* may also indicate such deep-seated reversals of asymmetry should be more carefully checked. Initial examination of the first post-basicoronal ambulacral plates revealed no deviations from conformity with Lovén's (1874) law (*cf.* p. 104, Durham, 1955) that would suggest a reversed pattern. If all specimens or any suspected of being reversed were cut frontally or examined with a fluoroscope, it should be possible to determine the course traversed by the digestive tract and get the best evidence from internal anatomy.

The pattern of asymmetry around the central axes through the interambulacral areas is very strongly marked in areas 1, 4, 5, fairly strongly marked in area 2, and rather weakly marked in area 3. It is possible that the deviations from the usual arrangements here too might be symptomatic of the more deep-seated *"situs inversus."* In some respects this study may be considered as an extension of Durham's (1955) notable work, which owed a great deal to the earlier thinking of numerous workers, of whom Lovén (1874) and Jackson (1912) must be singled out as especially important. At the same time it is obvious that in the present work there are more new avenues of investigation suggested than problems completely solved. Workers desirous of making additional studies of variation in irregular urchins should, in addition to the approaches used here, become thoroughly acquainted with the methods of Kongiel (1938), Kermack (1954), Nichols (1959a, 1959b, 1962) and Kier (1962).

SUMMARY

1. The general arrangement of plates on the oral surface of sand dollars is discussed.

2. Variations in this arrangement as they occur in *Echinarachnius parma* from several New England localities are indicated.

3. As this sand dollar increases in size, there is decreasing contact between the post-basicoronal interambulacral areas and the basicoronal plates.

4. The usual sequence in which this contact is lost among the areas is 5, 1, 4, 2 and 3, but all possible combinations of areas "out of contact" have been observed, except 2, 3 and 4.

5. The average numbers of areas "out of contact" for animals of comparable sizes vary among localities.

6. The asymmetry of loss of contact within the interambulacral areas has also been found to be highly variable.

7. The possibility that these variations may be related to differential environmental effects upon the rates at which different parts of the growth process occur is suggested.

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CHROMOSOMES OF TWO SPECIES OF QUAHOG CLAMS AND THEIR HYBRIDS

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Two species of quahogs (clams of the genus *Mercenaria*, formerly *Venus*) occur along the Atlantic and Gulf coasts of North America. Abbott (1954) characterizes the two species as follows: The northern quahog, *Mercenaria mercenaria* (L.), ranges from the Gulf of St. Lawrence to Florida and the Gulf of Mexico. It has a characteristic smoothish or glossy area on the exterior center of the valves. The interior of the valves is white and commonly has purple stainings. The entire lunule is three-fourths as wide as long. Two subspecies are listed: *M. m. notata* Say with external zigzag brown mottlings and *M. m. texana* Dall, from the northern Gulf of Mexico, with large irregular coalescing flat-topped concentric ribs. The southern quahog, *M. campechiensis* (Gmelin), ranges from the Chesapeake Bay to Florida, Texas, and Cuba. It has a more obese shell and lacks the smooth central area on the exterior of the shells. The entire lunule is usually as wide as long. Rarely are there brown mottlings on the exterior of the valves, which are always white internally.

It is often difficult to assign a specimen to either species if a single character is considered. Fast-growing specimens of *M. mercenaria*, less than about 25 mm. long, lack the characteristic glossy smooth area on the exterior of the valves. Measurements of length and weight of the two species, grown under the same conditions, have shown the small *M. mercenaria* to have heavier shells than *M. campechiensis* of the same length. Otherwise typical individuals of *M. campechiensis* occur with internal purple shell stainings and with the brown mottlings of the subspecies *M. m. notata*. Often the lunule of *M. campechiensis* is only three-fourths as wide as long.

The two species hybridize readily in the laboratory (Loosanoff, 1954). This paper reports chromosome numbers and behavior in the two species and their hybrids at meiosis and early embryonic mitoses.

MATERIALS AND METHODS

Live specimens of *M. mercenaria* were secured from Connecticut, New York, Delaware, Virginia, North Carolina, South Carolina and the east coast of Florida. The southern quahog was obtained from North Carolina, the east coast of Florida and several localities along the Gulf coast of Florida from Tampa Bay northward. These clams have been used in several ways: for growth experiments (Menzel, 1961a, 1962); for clam farming observations (Menzel, 1961b; Menzel and Sims, 1962); and as brood clams for observations on hybrids (Menzel, 1964). In addi-

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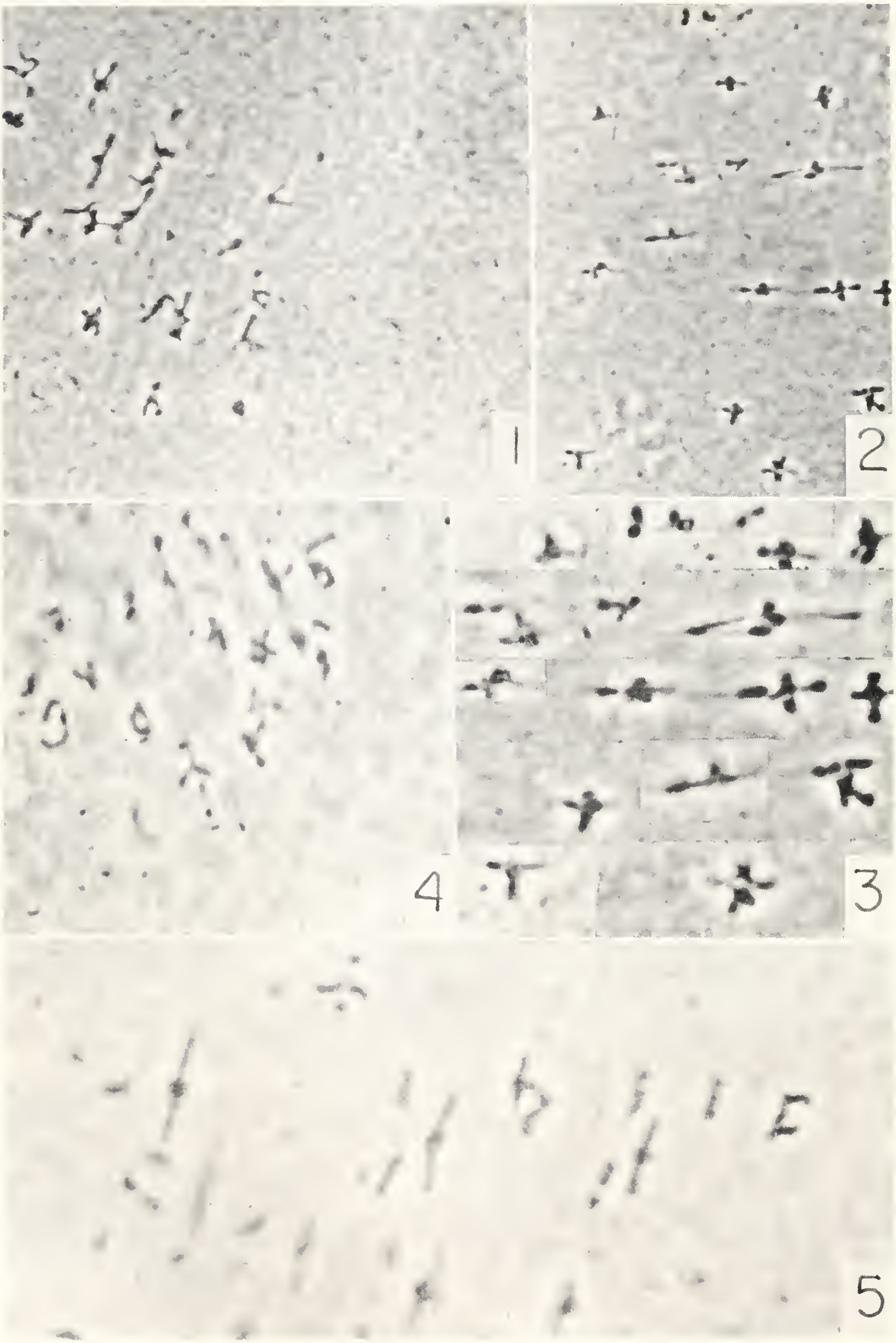
[1] Contribution No. 207 from Oceanographic Institute, The Florida State University, Tallahassee, Florida.



FIGURES 1–5.

tion, laboratory-reared hybrids have been available from the Biological Laboratory, Bureau of Commercial Fisheries, Milford, Connecticut, and from the marine laboratory of the Oceanographic Institute.

Crosses have been made in our laboratory using as brood clams hybrids grown to sexual maturity here and the two species from localities listed above. These crosses include intraspecific crosses, reciprocal crosses of the two species (F_1's), F_2's of the hybrids ♀ *M. campechiensis* × ♂ *M. mercenaria* and reciprocal backcrosses of the latter F_1 to each species. All of the above combinations have been spawned and reared beyond metamorphosis and settling by the techniques of Loosanoff and Davis (1963). Observations of meiosis in eggs from F_1 hybrids reported here were all made on hybrids from crosses between *M. mercenaria* males from Connecticut and *M. campechiensis* females from Florida.

At intervals after spawning, the eggs and embryos were fixed in freshly mixed acetic alcohol (three parts absolute ethanol, one part glacial acetic acid) and stored in a freezer at −16° to −18° C. Several dozen embryos in a small drop of fixative were placed on a slide and air-dried or flamed. Several drops of iron-acetocarmine were added and allowed to stain for two minutes. A coverslip was added and excess stain removed by blotting. The coverslip was pressed firmly on the slide and the preparation was then heated judiciously over an alcohol flame to clear the cytoplasm and further flatten the eggs. Such temporary squashes usually were examined at once with a Zeiss microscope equipped with an apochromatic optical system and phase contrast accessories, but they could be stored for several days at 2°–4° C. without severe deterioration.

Chromosomes prepared in this way were usually well spread but rather lightly stained. Substitution of aceto-orcein, propio-orcein, Gomori's chrom-alum-hematoxylin and Feulgen staining did not result in significantly better preparations. Phase-contrast illumination of the acetocarmine slides was used routinely to enhance contrast and facilitate analysis. Stages of meiosis from metaphase I to telophase II and mitotic figures from early cleavage divisions were readily observed by this method. Because of the dense cytoplasm and tough egg membranes, the eggs were difficult to flatten sufficiently for microphotography. Hence, most of the stages described here are illustrated with drawings made with the aid of a camera lucida.

Preparations from *M. campechiensis* were consistently better than those from the F_1 hybrid, which were in turn better than those from *M. mercenaria*.

OBSERVATIONS

Early embryology

Eggs fixed from 15 seconds to 5 minutes after spawning contained oocyte nuclei at metaphase I regardless of whether sperm suspension had been added. If the eggs were not fertilized, the oocyte nuclei remained at metaphase I for 60 minutes or longer and then gradually degenerated *in situ*. In one unfertilized lot of eggs,

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FIGURES 1–5. Photomicrographs of meiotic metaphase I in clam eggs, phase-contrast illumination. Some of the bivalents are out of focus in each photograph.

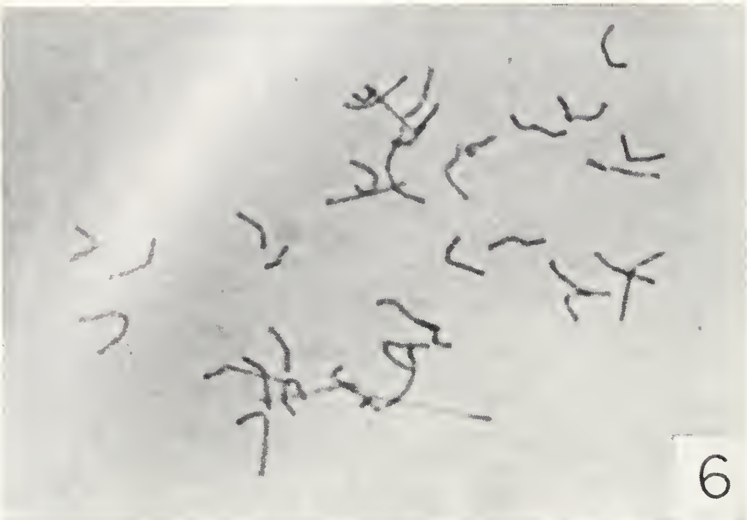
FIGURE 1. *Mercenaria campechiensis*, same nucleus as Figure 8, × 900.

FIGURE 2. *M. campechiensis*, same nucleus as Figure 10, × 900.

FIGURE 3. Individual bivalents of nucleus shown in Figures 2 and 10, × 1800.

FIGURE 4. F_1 hybrid, × 900.

FIGURE 5. *M. mercenaria*, same nucleus as Figure 12, × 1800.



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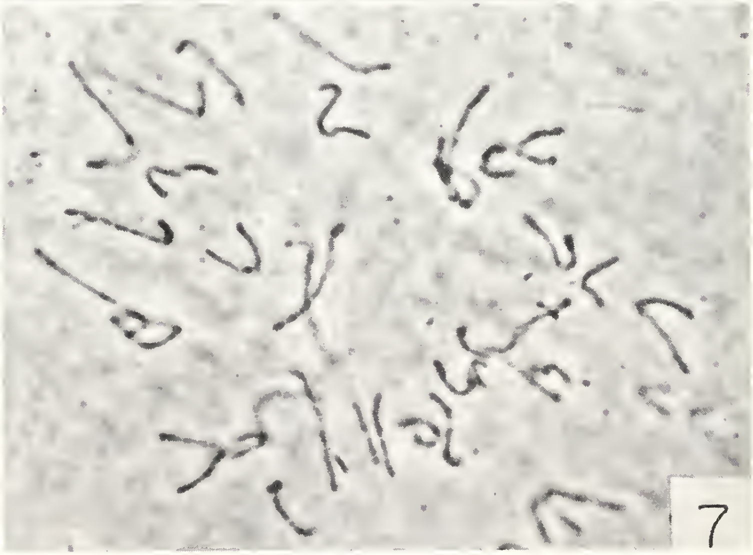

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FIGURE 6. Metaphase of the second cleavage division in a fertilized egg of *Mercenaria campechiensis*, 38 chromosomes. Acetocarmine staining, × 1800.

FIGURE 7. Metaphase of normal first cleavage division, F_2 hybrid, 38 chromosomes. Acetocarmine staining and phase-contrast illumination, × 1800.

recognizable though degenerating metaphase I configurations were present 20 hours after spawning. If an effective sperm suspension was added, meiosis proceeded rapidly, the first polar body appearing in 10 minutes, and metaphase II in 15 minutes. From metaphase I through telophase II the sperm pronucleus was discerned as an increasingly diffuse nucleus lying at some distance from the meiotic spindles. Fusion of the egg and sperm pronuclei was not identified with certainty but probably occurred when the chromosomes of both were in a mid-prophase condition preceding formation of the first cleavage spindle. The first cleavage division of the zygote nucleus ensued as early as 20 minutes and usually within 30 minutes after spawning. Subsequent cleavage divisions followed rapidly; eggs 75 minutes after spawning and contact with sperm suspension often contained too many dividing nuclei for analysis.

Systematic comparisons of the timing of development in the various types of fertilizations were not made. Preliminary observations suggested that fertilizations in which sperm from the F_1 hybrids were used (backcrosses to both parental species and F_2) were followed by somewhat delayed development. In one lot of F_2 embryos fixed on October 27, 1964, first cleavage metaphase and anaphase were found in lots fixed 45–75 minutes after sperm contact. Among 18 embryos in which chromosomes could be counted, 5 were diploid, 4 triploid (Fig. 16), 7 tetraploid, one had a chromosome number (46) between diploid and triploid, and one egg had a diploid and a separate haploid nucleus, both at metaphase. Subsequent lots of eggs from similar fertilizations did not exhibit polyploidy (Fig. 7). Occasionally an early embryo with dividing haploid nuclei was observed in batches of eggs which had not been fertilized either because sperm were not added or because the sperm were ineffective. A careful comparison of rates of development under controlled conditions should be made.

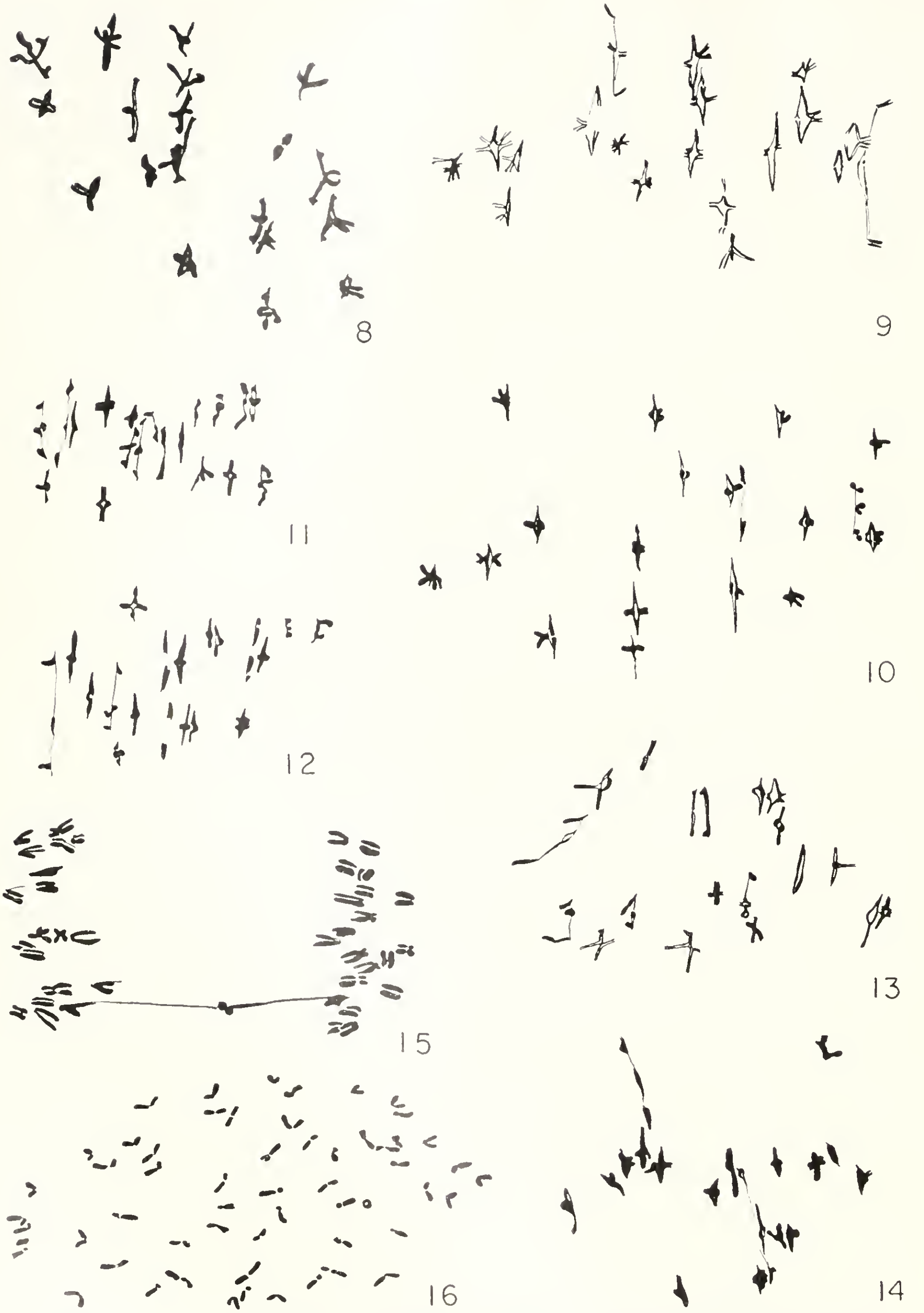
Chromosomes

Both *M. mercenaria* (Figs. 5, 11, 12) and *M. campechiensis* (Figs. 1–3, 8–10) have 19 pairs of chromosomes at metaphase I and 38 chromosomes at embryonic mitoses (Fig. 6). At metaphase I the chromosome pairs are small and slender and the chromatid split can often be discerned. Most of the chiasmata do not terminalize until the onset of anaphase. A typical nucleus from *M. campechiensis* (Fig. 9) showed 19 bivalents with 27 unterminalized and 8 nearly or completely terminalized chiasmata (1.89 chiasmata per chromosome pair). The metaphase I bivalents of *M. mercenaria* are similar, but in our material tended to be more compact and hence less easily analyzed for chiasma frequency and position. The F_1 hybrid was intermediate in this regard, some figures approaching those of *M. campechiensis* in clarity (Figs. 4, 13, 14).

In the F_1 hybrid all the chromosomes were paired regularly as 19 homomorphic bivalents at metaphase I. The chiasma frequency was not conspicuously different from those of the parents.

Later stages of meiosis proceeded conventionally in the two species and in the hybrid. In one batch of eggs from the hybrid, two anaphase I figures showed an apparent bridge between the two groups of chromosomes, one of which is shown in Figure 15. Since no fragments were found, the bridges probably resulted from lagging separation of chiasmata rather than from crossing-over within a heterozygous inversion. At anaphase I in both species and the hybrid the chromosomes at one spindle pole were commonly more compact and darkly stained than those at the other. In our squashes we were unable to tell whether either the darker or lighter group was consistently destined to be included in the first polar body.

The mitotic chromosomes of the first and second cleavage divisions were rather long and very slender (Figs. 6, 7) but tended to become shorter and more compact, at least at metaphase, in later divisions. Because of the rather high chromosome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FIGURES 8–16. Chromosomes in fertilized clam eggs. Drawings made with the aid of a camera lucida; all × 900 except Figure 15, × 1125.

number and small cells, it was not practicable to count the chromosomes of individual nuclei after the second cleavage metaphase. The individual chromosomes exhibited a rather wide range of relative lengths and of arm length ratios. Since the meiotic bivalents of the hybrid revealed no evidence of structural differences between the two species, detailed comparisons of mitotic karyotypes were not made.

Discussion

The ease with which hybrids between *M. mercenaria* and *M. campechiensis* can be made experimentally and the existence in nature of forms which can be interpreted as intermediate suggest that a certain amount of gene flow may occur between the two taxa. The homology and regular behavior of the chromosomes of the two species revealed at meiosis in the F_1 hybrid demonstrate that there is no gross chromosomal barrier to such gene interchange.

Ability to exchange genes under experimental conditions does not, of course, imply that such interchange actually does occur in nature. Mayr (1963) has recently reviewed the mechanisms which may serve to keep populations separate even though they are sympatric in part of their range and can be successfully hybridized under experimental conditions. Porter and Chestnut (1960) suggested that in the region of Beaufort, North Carolina, where *M. mercenaria* is confined to inland bays and inlets and *M. campechiensis* to outer shallow neritic waters, the two populations may be effectively separated by differential tolerance to salinity. The preliminary observations of F_2 and back-crosses suggest also that embryos of the species may have an advantage in rate of development over the hybrid offspring under certain conditions.

Regardless of whether and to what degree hybridization and gene exchange between *M. mercenaria* and *M. campechiensis* occur in nature, results so far suggest that their hybrids could furnish an important source of variation for the selection of improved strains of clams for commercial production, especially for regions in which the commercially less desirable *M. campechiensis* is naturally better adapted.

The authors gratefully acknowledge initial financial support from the Graduate Research Council, Florida State University, and the assistance of Mrs. Clare Shier in preliminary cytological study. Numerous individuals are thanked for kindly furnishing brood clams.

Summary

Chromosome numbers of $n = 19$, $2n = 38$ are reported for *Mercenaria mercenaria, M. campechiensis* and their F_1 hybrids. Meiosis is normal in the hybrids

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FIGURES 8–10. Meiotic metaphase I in *Mercenaria campechiensis*, 19 bivalents, mostly with one or two unterminalized chiasmata. Figure 8 is the same nucleus as Figure 1; Figure 10, the same as Figures 2 and 3.

FIGURES 11, 12. Meiotic metaphase I in *Mercenaria mercenaria*, 19 bivalents. Figure 12 is the same nucleus as Figure 5.

FIGURES 13, 14. Meiotic metaphase I in the F_1 hybrid, 19 bivalents, the chiasma frequency not conspicuously different from that of the parents.

FIGURE 15. Anaphase I in the F_1 hybrid showing one bridge or (more likely) pseudobridge.

FIGURE 16. Metaphase of an aberrant triploid first cleavage division, F_2 hybrid, 57 unusually short chromosomes.

and yields no evidence of chromosome nonhomology or structural rearrangements between the two parents. The hybrids produce functional eggs and sperm which result in normal fertilization and early embryonic divisions in reciprocal backcrosses and at least some F_2's. No gross chromosomal barrier to gene exchange appears to exist between the two species.

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STUDIES ON THE OOPLASMIC SEGREGATION IN THE EGG OF THE FISH, ORYZIAS LATIPES. III. ANALYSIS OF THE MOVEMENT OF OIL DROPLETS DURING THE PROCESS OF OOPLASMIC SEGREGATION

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Ooplasmic segregation, which generally occurs following fertilization in fish eggs, leads to the formation of the blastodisc. Studies of this movement have been done by Spek (1933; *Corregonus, Salmo,* etc.), Roosen-Runge (1938; *Brachydanio*), Lewis (1949; *Brachydanio*), and Costello (1948; *Nereis*), etc.

Although much attention has been paid to the protoplasmic movement in the yolk or endoplasmic region, observations on the movement in the cortical protoplasmic layer (cortex) have been restricted to the eggs of *Brachydanio* (Roosen-Runge, 1938) and of *Gasterosteus* (Thomopoulos, 1953), neither of which have oil droplets in the cortex. In *Oryzias* eggs, the protoplasm and yolk are well separated before fertilization and oil droplets are dispersed in the former. On fertilization, the oil droplets move toward the vegetal pole at a speed which can be measured accurately.

The present paper deals with analysis of the pattern of the movement of oil droplets, both natural and injected, during the formation of the blastodisc in *Oryzias* eggs.

PART I. MOVEMENT OF NATURAL OIL DROPLETS

METHODS

After fertilization in *Oryzias,* evenly dispersed oil droplets in the unfertilized egg cortex migrate toward the vegetal pole, fusing with each other, and finally assemble around the vegetal pole, turning the egg upside down within the chorion by buoyancy. Therefore, to prevent the rotation of the egg during observation, the egg must be placed with the animal pole down from the beginning, and fertilized; it is photographed simultaneously from the animal, vegetal and lateral sides at intervals of two minutes. The photographs of the egg are magnified 100-fold and superimposed to trace the movement of the oil droplets.

In the polar views, the distance along the curved surface between the oil droplets and the animal or vegetal pole is calculated from the tracings of the photographs. In the side view, the equator of the egg is taken as a reference line and the distance of the oil droplets from the line is calculated. Since the tracings of the moving oil droplets are almost parallel to the longitude of the egg, sidewise shifts of the droplet are neglected (Fig. 1).

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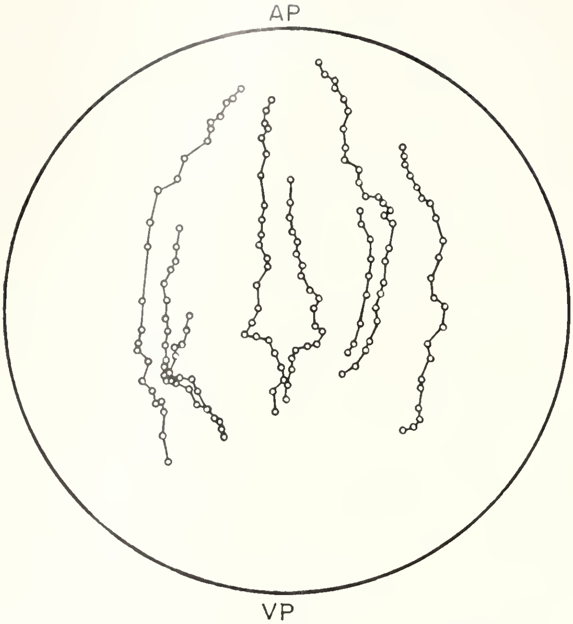

FIGURE 1. Tracings of moving oil droplets during ooplasmic segregation. AP: animal pole; VP: vegetal pole.

In the calculations, the egg is considered as a sphere. Since the egg is not strictly a sphere but rather an ellipsoid of revolution, the error coming from the approximation must be determined. In Figure 2, S is a sector of a sphere and E is that of an ellipsoid of revolution, a and b being the major and the minor axes of the

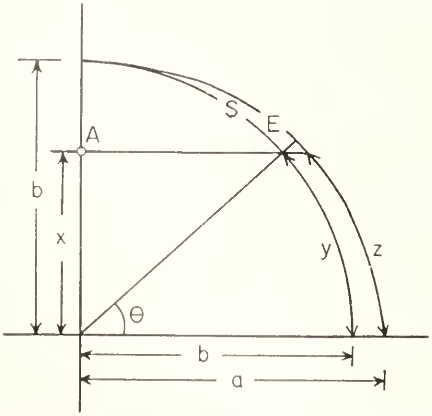

FIGURE 2. Procedure to obtain the actual distance or the angle θ from an apparent distance on the surface of sphere and of ellipsoid of revolution. S: a sector of a sphere; E: a sector of an ellipsoid of revolution; A: position of an oil droplet; a: major axis of ellipsoid of revolution; b: minor axis of the ellipsoid of revolution; x: apparent distance of an oil droplet from the reference line (equator); y: actual distance on the sphere; z: actual distance of x of the ellipsoid of revolution; θ: the angle at the center of the sphere embracing y.

latter. "A" represents the position of an oil droplet under observation and x is its apparent distance from the reference line, as expressed in an angle at the center embracing x; y is an arc of the circle and z is a section of the ellipsoid, which are the actual distances along the curved surfaces corresponding to the apparent distance x of the sphere and the ellipsoid of revolution, respectively. In other words, the aim of the calculation is to obtain either y or z from x.

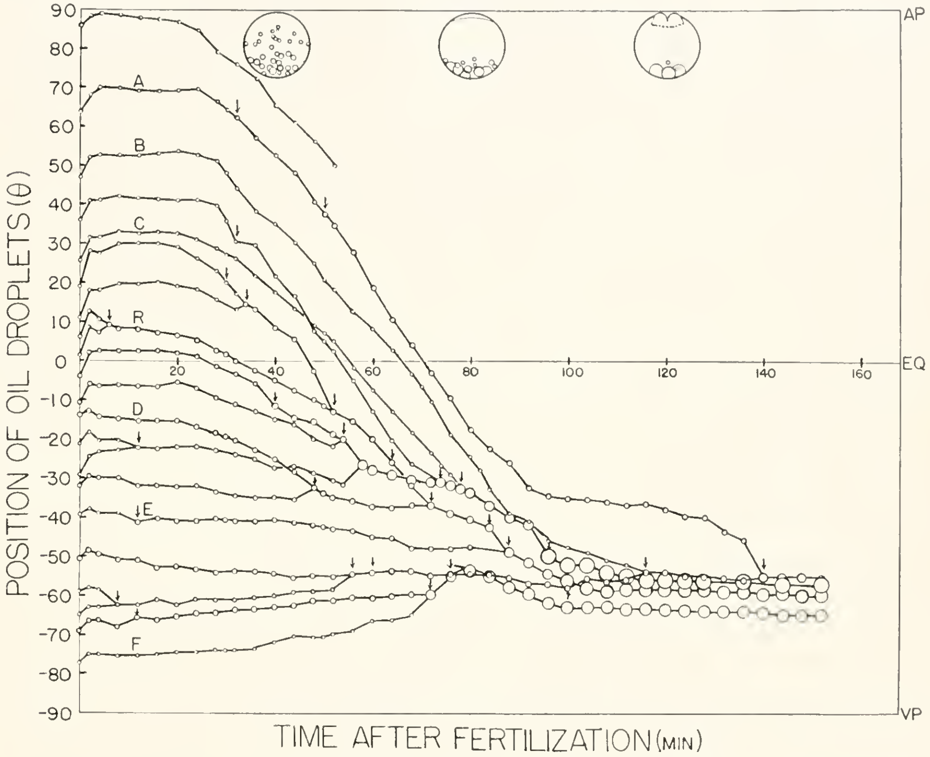

FIGURE 3. Time courses of the migration of oil droplets in terms of θ (22° C.). Ordinate: the value of θ, taking an equator as 0; abscissa: time after fertilization in minutes. AP: animal pole; VP: vegetal pole; EQ: equator; arrow: fusion of oil droplets. Alphabetic designation of the curves is for use in Figure 4.

In the *Oryzias* eggs used for the present measurements, the deviation of the ellipsoid from the sphere, $a-b/a$, is not larger than 0.08. For simplification of the calculation, $a-b/a$ is taken as 0.10. On the basis of these figures, the deviation of z from y, $z-y/z$, turns out to be less than 0.03, which means that the error involved in the approximation as a sphere is negligible. The measurement for the region above $\sqrt{3}/2$ b (60° in θ) is supplemented by the measurements in the polar views.

Since an egg can be treated as a sphere, distances through which the oil droplets move can best be expressed in θ because θ is independent of individual fluctuation in the egg size.

RESULTS

Figure 3 compares the time course of the migration in θ of oil droplets initially located at different regions of the egg. Within 2–4 minutes after fertilization, almost all the oil droplets shift transiently toward the animal pole to some extent (see the left end of Fig. 3). This shift is rather difficult to discern unless one is aware of this phenomenon beforehand. During this period, a decrease in the egg volume takes place in *Oryzias* as the result of the breakdown of cortical alveoli (T. Yamamoto, 1940). However, since the distance is expressed in θ, the decrease in the volume does not affect the measurement as long as the shrinkage occurs uniformly. Correspondingly to this stage when the animal region of the *Oryzias* egg is seemingly contracting, Roosen-Runge (1938) describes, in *Brachydanio* eggs, an increase of the egg diameter and flattening of the animal pole region, as observed from the upper side. According to the present writer's observations of *Brachydanio* eggs made at this stage from the side, the egg flattens and so does the blastodisc region. Whether or not this flattening of the blastodisc region means a contraction at the animal region cannot be said at present.

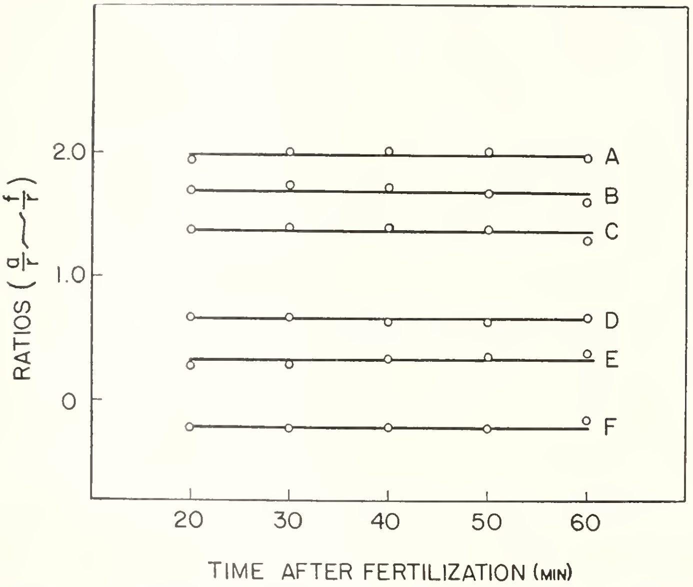

FIGURE 4. The ratio between the distance in θ of the reference oil droplet (R) from the base line (−60°) and the distance of a given oil droplet (A, B, C, D, E and F) from the line (−60°) at specified moments (a/r, b/r, c/r, d/r, e/r and f/r). Ordinate: ratios a/r, b/r, c/r, d/r, e/r and f/r; abscissa: time after fertilization.

In *Oryzias*, the oil droplets remain stationary thereafter for about 20 minutes, after which the movement of the oil droplets is resumed and they move toward the vegetal pole. This movement is particularly striking on the animal half.

On the other hand, the vegetal oil droplets, originally located within 30° in θ from the vegetal pole or beyond −60° on the ordinate of Figure 3, continue to move toward the animal side even after the transient shift is over. Consequently, all the droplets are assembled at the latitude of about −60° as a ring. It must be mentioned that the ring eventually reaches the vegetal pole after several hours, by the morula stage (omitted from Fig. 3).

If the migration speeds of the oil droplets starting from various levels of the egg are compared at various moments after fertilization, it can be said that the higher the curve, the steeper the inclination, which means that the closer an oil droplet is to the animal pole, the faster it moves.

Next, the latitude of −60° to which the oil droplets gather is taken as a base line, and the positions of seven droplets, A, B, C, D, E, F and R (Fig. 3) from the new base line are read in θ (a, b, c, d, e, f and r) for 20, 30, 40, 50 and 60 minutes after fertilization. In Figure 4, the ratios a/r, b/r, c/r, d/r, e/r and f/r at the specified moments are plotted. As is clear, the ratios for respective droplets remain almost unchanged during the migration. This means that these droplets approach the base line at a speed proportional to the original distance of the oil droplet from the base line, theoretically reaching the base line simultaneously, which is more or less what is observed.

The tracings of the oil droplets look as though the droplets might be attached to a stretched rubber membrane and carried toward −60° in θ by the snapping of the membrane, as the result of breaking at the two poles.

As is well known, the oil droplets frequently fuse with each other during the migration. When this happens, the speed of the fused droplet comes close to that of the slower or larger partner (see Fig. 3).

Discussion

Transient shift of oil droplets toward the animal pole immediately following activation

From the previous study by the author (Sakai, 1961), the unfertilized egg of *Oryzias,* deprived of the chorion, is flattened when observed from the side. On fertilization or activation, the egg is further flattened in the region where the cortical response is taking place. This flattening (decrease of the tension at the surface) spreads from the activated point with the wave of breakdown of cortical alveoli. After 2–4 minutes, when the cortical change is almost completed, the egg begins to bulge again from the activated point (Sakai, 1961).

In the fertilized egg enclosed within the chorion, while the tension at the surface is decreasing near the animal pole, the egg cortex on the vegetal side must still be adhering to the chorion because the cortical response has not yet taken place there. By the time the egg cortex at the vegetal pole detaches itself from the chorion with decreased tension, tension near the animal pole should have already begun to increase. If so, the egg cortex on the vegetal side must be pulled toward the animal pole and the transient shift of oil droplets toward the animal pole, mentioned in connection with Figure 3, becomes understandable.

Stationary phase

Within about 20 minutes after the completion of the cortical response, the naked egg bulges higher than before fertilization. This 20-minute period coincides with that of the stationary phase, so that it seems that oil droplets do not begin to move until the tension at the surface reaches a certain level. Similarly, if a part of the yolk is sucked out from the egg about 15–20 minutes after fertilization, when oil droplets would begin to migrate under natural conditions, the egg is flattened and the droplets in the treated egg do not migrate until the egg rounds up again. In such eggs having lost a part of the yolk, the formation of the blastodisc tends to be retarded and so does the accumulation of oil droplets at the vegetal pole. The recovery of the egg shape (recovery of the level of tension), therefore, seems to be essential for the initiation of the migration of oil droplets. These observations are of interest in connection with the fact that, in *Brachydanio* eggs, a protoplasmic movement inside the yolk and a counterstream in the protoplasmic coat begin only after the egg becomes exactly round (Roosen-Runge, 1938). However, no explanation is available concerning the manner in which a higher membrane tension and the bipolar segregation of the protoplasmic components are connected with one another.

PART II. MOVEMENT OF INJECTED OIL DROPLETS FOLLOWING ACTIVATION

After analyzing the movement of the natural oil droplets, it is of interest to see how a droplet of oil foreign to the egg will move when it is introduced into the egg by injection.

METHODS

Salad oil (as a neutral oil; acid value (A.V.) = 0.22), liver oil (as an acidic oil; A.V. = 0.52), and mineral oil (as a non-polar oil) were used as substances to be injected.

To distinguish the injected oil droplet from the natural ones of the eggs, the oil to be injected was previously stained with Sudan III. By using a micromanipulator, an oil droplet of a size similar to that of natural ones (20–70 μ in diameter) was injected into the cortical protoplasmic layer (cortex) of the unfertilized eggs, either centrifuged or non-centrifuged, and of the fertilized eggs at the one-, two-, and 8-cell stages. When oil droplets are injected into the fertilized eggs, the chorion is previously removed by using the hatching enzyme of *Oryzias* (Sakai, 1961). Since the cortex of the unfertilized eggs is very thin, the tip of the injection needle sometimes misses it. If the oil happens to be injected into the yolk, the oil moves freely by gravity. If the oil is injected at too shallow a layer, the oil is apt to be squeezed out of the egg surface into the perivitelline space after the alveolar breakdown. As a result, successful injection can easily be determined.

For measuring the movement of the injected oil droplet, the same procedure is applied as that which was used in Part I.

RESULTS AND CONCLUSION

Behavior of the injected oil droplet

The oil of *Oryzias* eggs is a kind of neutral oil because it is stained deeply with Sudan III and Sudan black, and also stained a pink color with Nile blue sulfate at about pH 7.0. Such a stainability is the same as that of oil droplets of *Onchorynchus* eggs (K. Yamamoto, 1958).

To test another kind of neutral oil, salad oil is used. After the injection of salad oil, the eggs are fertilized or activated by pricking. Although some eggs are activated by the injection procedure itself, the behavior of the injected oil is much the same as that in the eggs activated after a successful injection in an inactivated condition.

When injected at the equatorial region, the injected oil, on pricking, generally migrates toward the vegetal pole, fusing with the natural oil during the movement (Fig. 5). In fertilizable but slightly under-ripe eggs, merging of the natural oil droplets among themselves rarely occurs but even under such a circumstance, the injected oil droplets move to the pole side by side with the natural ones. Occasionally, the injected oil fails to move, probably owing to imperfection of injecting technique, in spite of the successful migration of natural oil droplets lying closer to the animal pole than the injected one. In such cases, natural droplets situated on the animal half overtake the injected oil and pass it closely around its circumference. However, if the oil droplet migrates at all, it always migrates toward the vegetal pole, and never toward the animal pole. Quantitatively, too, the behavior of the injected oil corresponds well to that of the natural droplets as shown in Figure 6. The frequency of the migration is less when the oil is injected close to the animal pole than when injected at the equator.

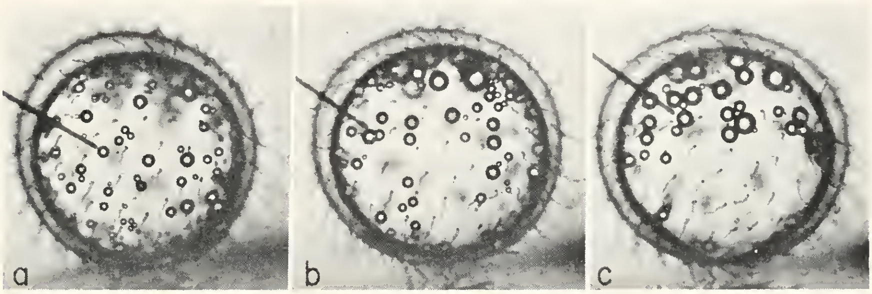

FIGURE 5. Movement of oil droplet injected at the equatorial region of the unfertilized egg (side view). The egg is placed upside down to avoid the rotation of the egg caused by assembling oil droplets. (a) 20 minutes after fertilization; (b) about 40 minutes after fertilization; (c) one hour after fertilization. Injected oil has fused with the natural oil.

To clarify whether or not the behavior of the injected oil varies with its properties, similar experiments were repeated by using liver oil, mineral oil and the oil of *Oryzias* eggs. These experiments give substantially the same results as that of the salad oil. Judging from the fact that the injected oil of *Oryzias* itself sometimes fails to move, it is most likely that the failure is not due to the properties of the oil but to disturbance caused by the injection technique.

Relationship between movement of protoplasm and oil droplets

From the foregoing results, the oil droplets migrate irrespective of the nature of the oil itself. However, this still leaves the possibility open that the migration of the oil droplets is somehow coupled with the movement of the protoplasm. To make sure of this point, the following two conditions were tested: (1) weakly centrifuged eggs (100–200 *g* for about three minutes), with the natural oil droplets shifted to one side but leaving the protoplasm undisturbed, (2) strongly centrifuged eggs (900–1800 *g* for 10 minutes), with both oil and protoplasm localized at the opposite sides.

In non-injected eggs, shifting of the natural oil by weak centrifugation does not interfere with ooplasmic segregation, regardless of the abnormal localization of the droplets after centrifuging. In centrifuged eggs, the injection is made where the natural oil droplets are no longer found, although protoplasm and cortical alveoli are still present in the egg cortex. In spite of the absence of the natural oil droplets around the injected oil, it can migrate toward the vegetal pole all by itself.

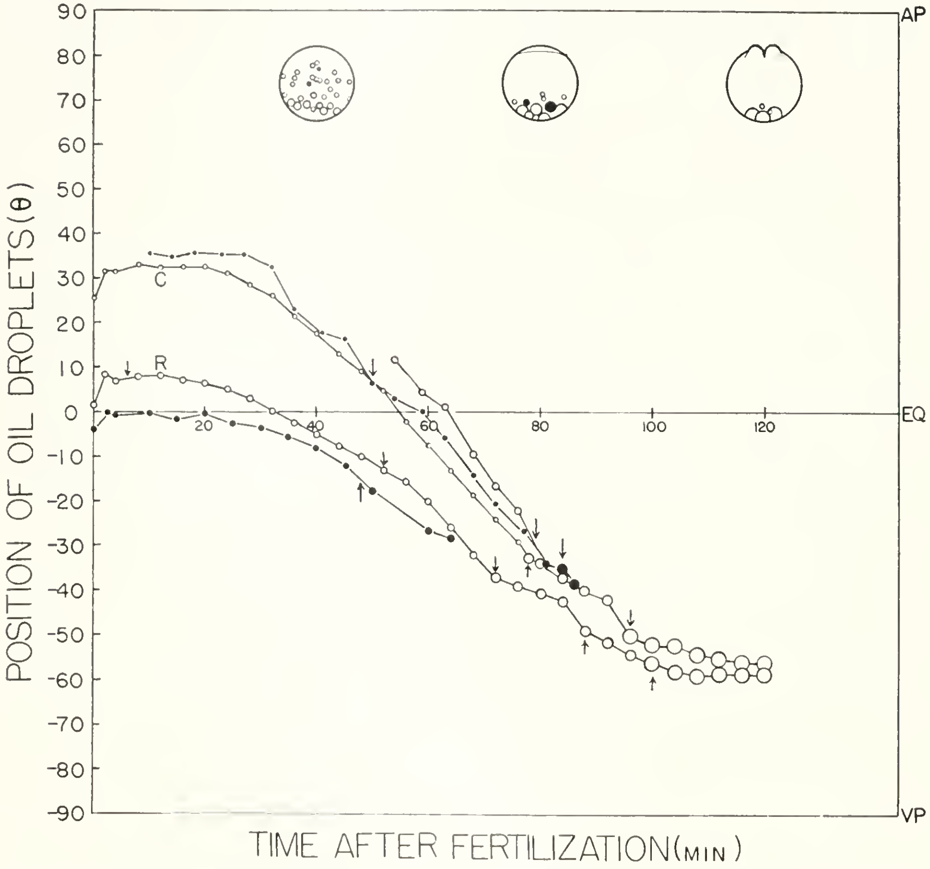

FIGURE 6. Time courses of the migration of injected oil droplets in comparison with the natural droplets. Ordinate: the value of θ, taking the equator as 0; abscissa: time after fertilization in minutes; closed circle: injected oil droplets; open circles: natural oil droplets; AP: animal pole; VP: vegetal pole; EQ: equator; arrow: fusion of oil droplets.

On the other hand, by strong centrifugation, the protoplasm of the unfertilized egg can be shifted in the animal region and the oil is massed at the vegetal pole by orienting the egg by a capillary tube. As the result, a blastodisc is formed at the centrifugal animal side where the protoplasm has already collected. The oil is injected at the equatorial region where little protoplasm is found. Under these conditions, the injected oil is fixed at the injected point and never migrates toward the vegetal pole within the observation period of three hours.

To further confirm the idea that the migration of oil droplets is caused by the movement of the protoplasm, the oil is injected near the equator of the egg at the one-, two- and 8-cell stages in which the protoplasmic segregation has almost been completed. The injected oil droplets never migrate toward the vegetal pole. The relationship between oil and protoplasm is also pointed out by the following results.

When an egg is forced into a capillary, both the migration of oil droplets and the formation of the blastodisc are much delayed. Furthermore, if more than one protoplasmic accumulation is induced by polyspermy or strong prickings, such protoplasmic accumulations are always accompanied by the migration of oil droplets toward the opposite side of each accumulation (Sakai, 1964a). Further, the experiments on partial activation indicate that the oil migration does take place only in the activated half (Sakai, 1964b).

If the migration of the protoplasm has a leading role over the movement of natural oil droplets, the elimination of the oil droplets is expected to have no influence on the movement of the remaining protoplasm. In the eggs weakly centrifuged at 100–200 *g* for about 5 minutes just after the fertilization, the cortex protrudes where the oil is forced to gather. Such a mass of oil can be sucked out with a micropipette. As in *Nereis* egg fragments observed by Costello (1940), yet the migration of the protoplasm can still occur and form the blastodisc.

On the other hand, careful observation reveals that the protoplasmic movement always precedes that of the oil droplets, that is, by the end of the stationary phase, a small amount of protoplasm has already begun to accumulate at the animal pole, slightly flattening the yolk surface under it.

Considering the above-mentioned results in connection with this observation, it will be concluded that the migration of oil droplets is a consequence of the movement of protoplasm toward the animal pole.

The author is indebted to Professor K. Dan for his invaluable advice. The author's thanks are also due to Dr. M. Yoneda for his kind help in the calculations.

SUMMARY

The movement of oil droplets in *Oryzias* eggs, natural and artificially injected, was analyzed during ooplasmic segregation. (a) During 2–4 minutes after fertilization, natural oil droplets are shifted transiently toward the animal pole, followed by a stationary phase of about 20 minutes. After this phase, all of the oil droplets coming either from the animal or from the vegetal side assemble at about −60° below the equator as a ring and later reach the vegetal pole. The migration is faster in droplets coming greater distances than in those coming shorter distances. (b) The pattern of the migration of injected oil droplets is the same as that of the natural ones, irrespective of their nature. The migration is possible in weakly centrifuged eggs in which the protoplasm remains undisturbed in the cortex.

However, injected oil droplets no longer move after shifting of the protoplasm by strong centrifugation or after the completion of ooplasmic segregation.

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GROWTH AND SURVIVAL OF POSTLARVAL PENAEUS AZTECUS UNDER CONTROLLED CONDITIONS OF TEMPERATURE AND SALINITY[1]

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Temperature and salinity may be considered among the most important abiotic factors influencing the growth and survival of much of the estuarine fauna. They are of particular significance to those organisms that spend certain portions of their life cycle in the open sea where both factors are relatively stable, and other portions in the estuarine areas where both temperature and salinity may change drastically. Although temperature is generally thought to overshadow salinity in its effects on migratory organisms, salinity, probably through its osmotic effects, also plays a part in limiting some organisms to specific environments.

Several investigators have attemped to evaluate the importance of temperature and salinity to penaeid shrimp of the Gulf of Mexico, but ecological questions concerning these factors remain unanswered. Although field studies have dealt with the relationship of shrimp to salinity, the conclusions reached have differed widely enough to warrant further investigation. The interpretation of observations on salinity and shrimp abundance in nature is made difficult by changes in other environmental factors, some of which frequently vary with salinity. Such factors include temperature, light, substrate, food supply, cover and pollution. For this reason, controlled-environment studies in the laboratory were employed in the present work.

In an earlier study, Zein-Eldin (1963) determined that under conditions of constant temperature and somewhat restricted food supply, grooved *Penaeus* postlarvae[2] survived and grew over a wide range of salinity (2–40‰). However, it has been suggested that in other migratory Crustacea, notably in the European shrimp, *Crangon crangon* (Broekema, 1941), as well as in juvenile and adult brown and pink shrimp, *Penaeus aztecus* and *P. duorarum,* respectively (Williams, 1960), temperature can influence tolerance to salinity. Thus, further studies were designed to test the combined effects of temperature and salinity on the survival and growth of postlarval brown shrimp.

MATERIALS AND METHODS

The work was of two types, 24-hour survival studies and a 28-day growth experiment. For all work, postlarval brown shrimp of approximately 12 mm. rostrum-telson length were seined from the Gulf of Mexico surf at the entrance to Galveston Bay. The animals were held in the laboratory in aerated water of approximately 25‰ and 25° C. for at least 24 hours prior to use. Few mortalities occurred during this preliminary holding period.

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[1] Contribution No. 205, Bureau of Commercial Fisheries Biological Laboratory, Galveston, Texas.

[2] As defined by Renfro (1964).

The first objective was to obtain a rapid, crude estimate of postlarval tolerance to salinity and temperature in order to provide guidelines for the more detailed and sensitive growth study to follow. Accordingly, we selected short-term survival as a rough index suited to our needs.

To determine the short-term tolerance of brown shrimp to salinity and temperature, we exposed groups of experimental shrimp to different levels of the two factors for 24 hours. The test levels were chosen to include and extend above and below the ranges of salinity and temperature at which large numbers of postlarvae have been observed in nature (Bearden, 1961; Williams, 1955; Baxter, unpublished).

Temperature control of ±0.5° C. was maintained by B.O.D.-type incubators. Salinity changes were effected by replacing portions of water in the test containers with equal volumes of either distilled water or evaporation-concentrated sea water. Salinity was determined by hydrometer and reported to the nearest part per thousand. Four series of 24-hour survival experiments were carried out with groups of 5 to 30 animals as described below. Series 1, 2 and 4 had an initial salinity of 24–25‰ and an initial temperature of 24° to 26° C., matching conditions of the holding aquaria. Initial salinity in Series 3 was 40‰, equal to the unusually high level at which animals for that series were collected. Following introduction of the shrimp into the vigorously aerated experimental containers, stepwise changes in both temperature and salinity (0 to 8 steps, depending on the magnitude of change involved) were made over a 10- to 12-hour period, to reach the desired conditions. The attainment of these conditions marked the beginning of the 24-hour test period. At the end of that time, the beakers were removed from the incubators and the live and dead postlarvae counted. Failure to show either spontaneous or probe-induced activity upon return to room temperature was considered indicative of death. No food was provided during the experiment.

Previous observations of postlarvae in the laboratory had indicated that failure to keep them under water mechanically could lead to considerable mortality, due to their jumping activity. This type of loss was avoided during the first two series by restraining each group of animals in a one-liter beaker whose mouth was filled with a stemless funnel 4 inches in diameter. When the beaker was filled with water, all air space accessible to the shrimp was eliminated, thereby preventing the animals from escaping the vessel or adhering to its dry surfaces. Aeration was provided by means of an air stone attached to $\frac{3}{16}$-inch O.D. Tygon tubing fitted through the hole in the funnel.

In the first two series, we employed only five shrimp per group, hoping thus to reduce cannibalism. Although losses caused by escape from the water were successfully avoided, test results indicated that cannibalism occurred at intermediate and higher temperatures where the number of survivors plus the number of dead animals remaining per group frequently totaled less than the original number of shrimp (Table I). In such cases, we attempted to discriminate between deaths due to cannibalism and mortalities attributable to salinity and temperature by arbitrarily assuming that salinity-temperature combinations causing the rapid death of some experimental shrimp were sufficiently severe to inhibit feeding, including cannibal-

TABLE I

Survival of P. aztecus postlarvae after 24-hour exposure to various combinations of salinity and temperature

Series no.	Shrimp per group	Acclimated for: (hrs)	Experimental conditions																									
1	5	10–12	Temperature (° C.)	7					15					25					30					35				
			Salinity (‰)	3	8	23	32	36	5	10	24	34	37	5	11	25	34	37	6	10	26	36	41	7	11	27	36	37
			Survivors	0	1	5	5	4	1	4	5	5	5	3	4	5	5	5	4	4	4	5	4	1	2	1	1	3
			Intact dead*	5	4	0	0	1	4	1	0	0	0	0	0	0	0	0	0	1	0	0	0	4	1	2	3	1
2	5	10–12	Temperature (° C.)	7					15					25					30					36				
			Salinity (‰)	5	9	24	34	38	4	9	24	34	38	4	8	24	34	38	4	9	24	33	37	4	9	25	34	39
			Survivors	0	2	5	5	4	0	3	5	5	5	4	4	5	5	4	3	3	5	4	5	0	1	1	3	2
			Intact dead*	5	3	0	0	1	5	2	0	0	0	0	0	0	0	1	0	0	0	0	0	5	3	4	0	2
3	10	10–12	Temperature (° C.)	7					15					25					31					34				
			Salinity (‰)	6	10	25	36	37	7	11	26	34	38	7	10	26	35	39	6	11	26	34	39	7	11	26	35	39
			Survivors	0	6	10	10	10	8	10	10	10	10	8	10	10	10	10	10	10	10	10	10	9	9	10	10	10
4	30	10–12	Temperature (° C.)				10									25							34					
			Salinity (‰)				10		39							25							11		41			
			Survivors				26		28							30							28		30			
—	30	0	Temperature (° C.)				10																34					
			Salinity (‰)				11		39														11		41			
			Survivors				5		0														18		26			

* These were dead shrimp which showed no signs of having been eaten by the other experimental animals within the group. See text.

ism, among the survivors. High rates of penaeid activity, including movement, feeding, and molting, have been observed at temperatures of 25° and 32° C. in this laboratory. Such activities favor cannibalism among shrimp under relatively crowded experimental conditions. On this basis, we counted as mortalities only those dead animals visibly present at the end of each 24-hour experimental period.

To test this assumption, we required data unaffected by cannibalism. These were obtained in Series 3 by confining each animal within a $1\frac{1}{2}$-inch length of 14-mm. Pyrex tubing, both ends of which were covered with cotton gauze held in place with small rubber bands. While permitting contact between the animal and its experimental aquatic environment, this procedure prevented "jump-out" losses and made physical contact between shrimp impossible. Ten postlarvae thus isolated were placed in each test beaker and the experimental conditions established as before. The survival results agreed well with those of the two earlier series as interpreted above, tending to substantiate our assumption regarding the effect of cannibalism.

TABLE II

Schedule of salinity and temperature changes [initial salinity and temperature were 26‰ and 23° C., respectively]

Elapsed time (hr.)	Desired salinity (‰)					Desired temperature (° C.)			
	2	5	15	25	35	11	18	25	30
	Actual salinity					Actual temperature			
2	20	20	20	25	25	23	23	23	26
8	15	15	15	25	30	19	22	24	30
24	10	10	15	25	35	17	19	25	33
36	5	5	15	25	35	12	18	25	33
48	2	5	15	25	35	11	18	25	32

In a fourth series, further survival data were sought at temperature-salinity combinations which seemed to be near the extremes of postlarval tolerance, as suggested by results of the three previous series (Table I). In this series the importance of acclimation was also estimated. Each set of experimental conditions was duplicated in two two-liter beakers, one for shrimp acclimated as usual, the other for animals which were transferred directly from the holding tank to the extreme salinity and temperature levels to be tested. Thirty individually confined postlarvae were held in each beaker, and 24-hour survival determined as before.

For the growth study, 46 liters of brackish water and 100 animals were placed in each of twenty 15-gal. aquaria. Filtration, aeration, and confinement were accomplished as previously described (Zein-Eldin, 1963). Five aquaria were placed in each of four constant-temperature rooms. The experimental temperatures were changed from the initial 23° C. to 11°, 18°, 25°, or 32° C. Water temperature, although ±0.5° C. in a given aquarium, varied as much as 1° C. among aquaria in a single room. The initial salinity of 23‰ was simultaneously adjusted stepwise with temperature, to final levels of 2‰, 5‰, 15‰, 25‰, or 35‰ (Table II). Each tank was continuously illuminated by two 40-w. fluorescent lamps.

Postlarvae were fed live brine shrimp (*Artemia*) nauplii throughout the growth experiment. The nauplii in a 0.1-ml. sample of brine shrimp in water were counted to estimate number per unit volume, and the volume of food recorded at each feeding of the postlarvae. *Artemia* nauplii were filtered and washed with distilled water before their addition to the tanks, in order to avoid increases in experimental salinity levels. Live brine shrimp nauplii were present in excess in all aquaria during the first 24 days of the experiment. During the last four days at 32° C., however, the shrimp had grown to such a size that maintaining an excess food supply became almost impossible, even though 400,000 to 500,000 nauplii per tank (a minimum of 9000 to 10,000 per experimental animal) were supplied per day.

TABLE III

Cumulative mortality [only observed deaths are included]

Elapsed time (days) — Temperature (° C.)	32					25					18					11				
Salinity (‰)	2	5	15	25	35	2	5	15	25	35	2	5	15	25	35	2	5	15	25	35
1	5	1	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0
2	41	25	16	2	0	0	0	0	0	0	2	2	0	0	0	4	2	0	0	0
3	44	25	16	6	0	0	0	0	0	0	13	3	0	0	0	77	2	0	0	0
4	45	25	17	7	0	0	0	0	0	0	17	3	0	0	0	93	5	0	0	0
5	45	25	17	7	0	0	0	0	0	0	19	3	0	0	0	97	12	0	0	0
6	45	25	17	7	0	0	0	0	0	0	19	3	0	0	0	97	12	0	0	0
7	45	25	17	7	0	0	0	0	0	0	19	3	0	0	0	97	22	0	0	0
8	45	25	17	7	0	0	0	0	0	0	19	3	0	0	0	97	25	0	0	0
9	45	25	17	7	0	0	0	0	0	0	19	3	0	0	0	97	32	0	0	0
10	45	25	17	7	0	0	0	0	0	0	19	3	0	0	0	97	37	0	0	0
11	45	25	17	7	0	0	0	0	0	0	24	3	0	0	0	97	41	0	0	0
12	45	25	17	7	0	0	0	0	0	0	26	4	0	0	0	97	45	0	0	0
13	45	25	17	7	0	0	0	0	0	0	27	4	0	0	0	97	50	0	0	0
14	45	25	17	7	0	0	0	0	0	0	27	4	0	0	0	97	55	0	0	0
17	45	25	17	7	0	0	0	0	0	0	29	4	0	0	0	97	60	0	0	0
18	45	25	18	7	0	0	0	0	0	0	31	4	0	0	0	97	60	0	0	0
19	45	25	18	7	0	0	0	0	0	0	45	4	0	0	0	97	60	0	0	0
20	46	25	18	7	0	23	0	0	0	0	57	4	0	0	0	97	63	0	0	0
21	46	25	18	7	0	39	0	0	0	0	58	5	0	0	0	97	63	0	0	0
22	46	25	18	7	0	50	0	0	0	0	58	5	0	0	0	97	63	0	0	0
24	46	25	18	7	0	55	0	0	0	0	58	5	0	0	0	97	66	0	0	0
28	46	25	18	7	2	55	0	0	0	0	58	5	0	0	0	97	67	0	0	4
No. of animals removed for measurement	25	30	35	50	50	40	50	50	50	50	36	50	50	50	50	0	30	50	50	50
No. escaped	2	0	0	0	0	4	4	3	0	0	1	2	2	2	0	0	0	0	0	0
Observed survivors	15	22	25	29	47	0	43	43	50	49	0	41	46	48	49	0	0	49	46	43
Unobserved deaths	12	23	22	14	1	1	3	4	0	1	5	2	2	0	1	3	3	1	4	3
Per cent survival	21	31	38	58	94	0	93	91	100	98	0	85	96	100	98	0	0	98	92	86

At approximately 5-day intervals, 10 animals were removed from each aquarium. These included both the largest and smallest specimens, and eight collected at random. The animals were individually measured to the nearest 0.5 mm., blotted dry, weighed to the nearest 0.1 mg. with a Mettler H15 analytical balance, and preserved. At the termination of the experiment, all remaining animals were similarly treated. The final per cent survival was determined by comparing the total number of shrimp remaining at the close of the experiment to the number that theoretically should have been present (original number less those that had been removed for sampling and a few that had escaped). The unobserved deaths recorded in Table III were animals not accounted for either as survivors, observed deaths, or those sampled for measurement.

On the assumption that an individual *Artemia* nauplius weighs an average of 7.1 μg. (D. Godwin, unpublished), we estimated conversion efficiency by comparing the calculated wet weight of the brine shrimp that were fed with the weight gain of the surviving penaeids. Determinations were made only for those temperature and salinity combinations at which survival was 85% or greater.

Although the design of the experiment was similar to that of Costlow, Bookhout and Monroe (1960, 1962) in studies of larval crab survival, we did not use the statistical methods which they employed. The fitted-surface method of Box and Youle (1955) has proved valuable in industrial applications of physical and chemical interactions whose principles are sufficiently well defined to permit relatively safe extrapolation from a limited number of experimental observations. However, the complex nature of biological responses to temperature and salinity renders such extrapolation extremely speculative. In the present study, we have tested a group of temperature-salinity combinations which represents a relatively large range of levels for each factor. Our interpretations of the results exclude extrapolation.

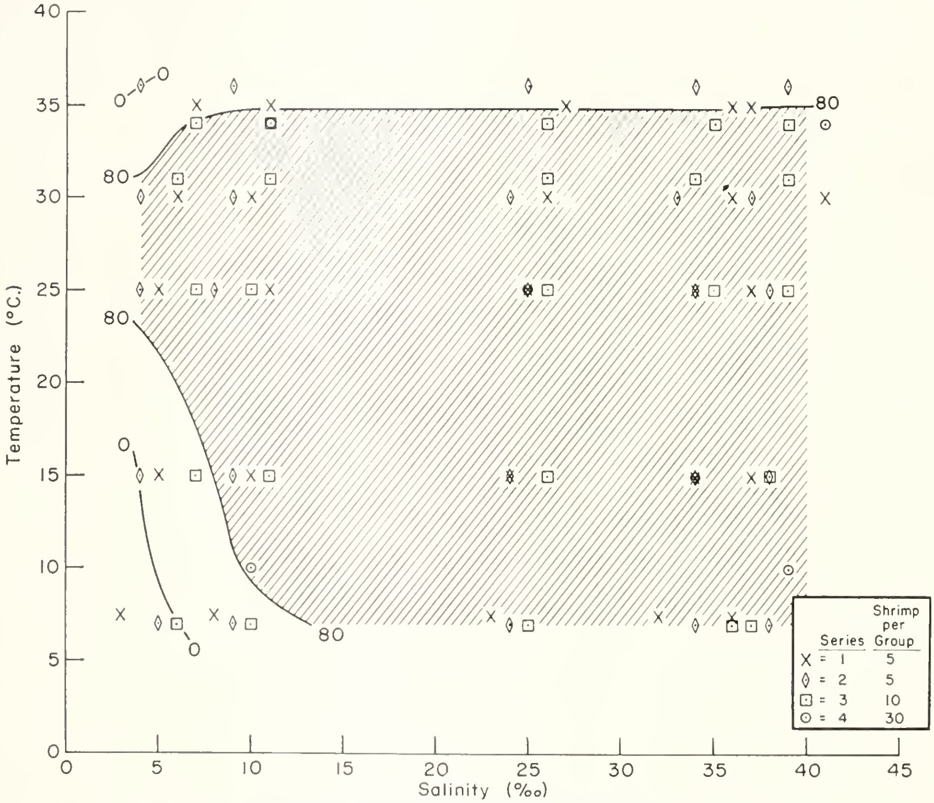

FIGURE 1. Per cent survival of *P. aztecus* postlarvae after 24 hours at indicated levels of salinity and temperature.

RESULTS AND DISCUSSION

Short-term survival

The excellent survival of postlarvae for periods of 24 hours under most of the experimental conditions suggested a broad zone of short-term tolerance to both salinity and temperature (Table I). The animals were quite euryhaline, especially at 25° and about 30° C., although a marked reduction in tolerance to salinity levels below 10‰ was demonstrated at 7° and 15° C. (Fig. 1). A general reduction in survival near 35° C., regardless of salinity, suggests a strong temperature effect. The absolute limiting (maximum) temperature for *P. aztecus* is probably only slightly above 35° C.

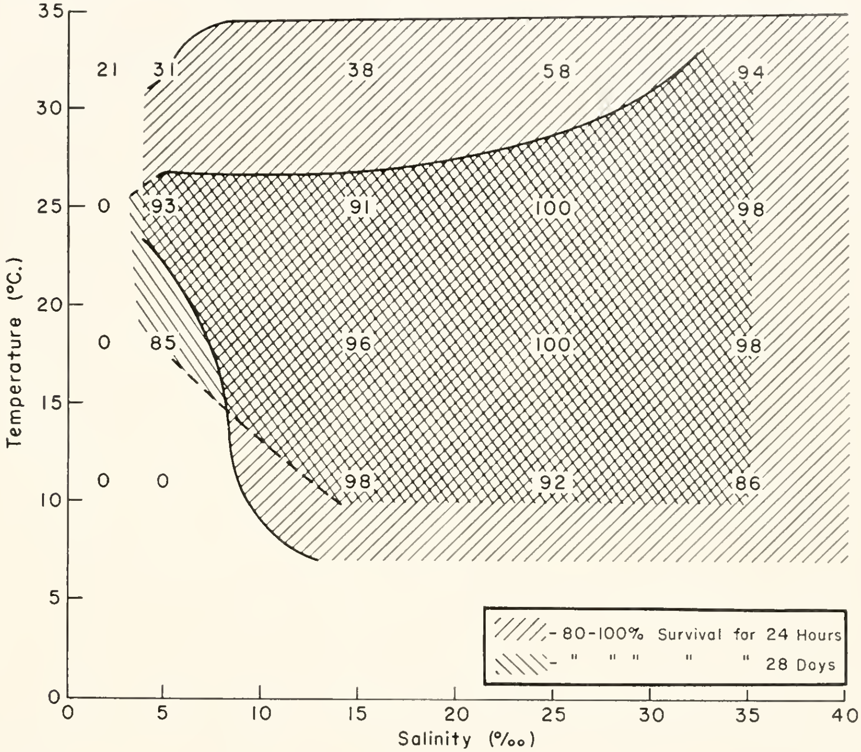

FIGURE 2. Long- and short-term survival of *P. aztecus* at indicated levels of salinity and temperature. Numbers indicate 28-day survival in per cent.

The effect of acclimation in extending ranges of postlarval tolerance is clearly shown in the results of the fourth series. In each of the four combinations of temperature and salinity, gradually changed conditions permitted better survival than did sudden changes (Table I). This effect was considerably more marked at 10° than at 35° C.

28-Day survival

The survival of postlarvae in the 28-day experiment further confirmed this wide zone of tolerance to salinity and temperature. Although the per cent survival for 28 days was somewhat lower than that observed for only 24 hours, in most cases the results were much the same (Fig. 2). There is some suggestion of greater long-term survival for animals at low temperature and low salinity than

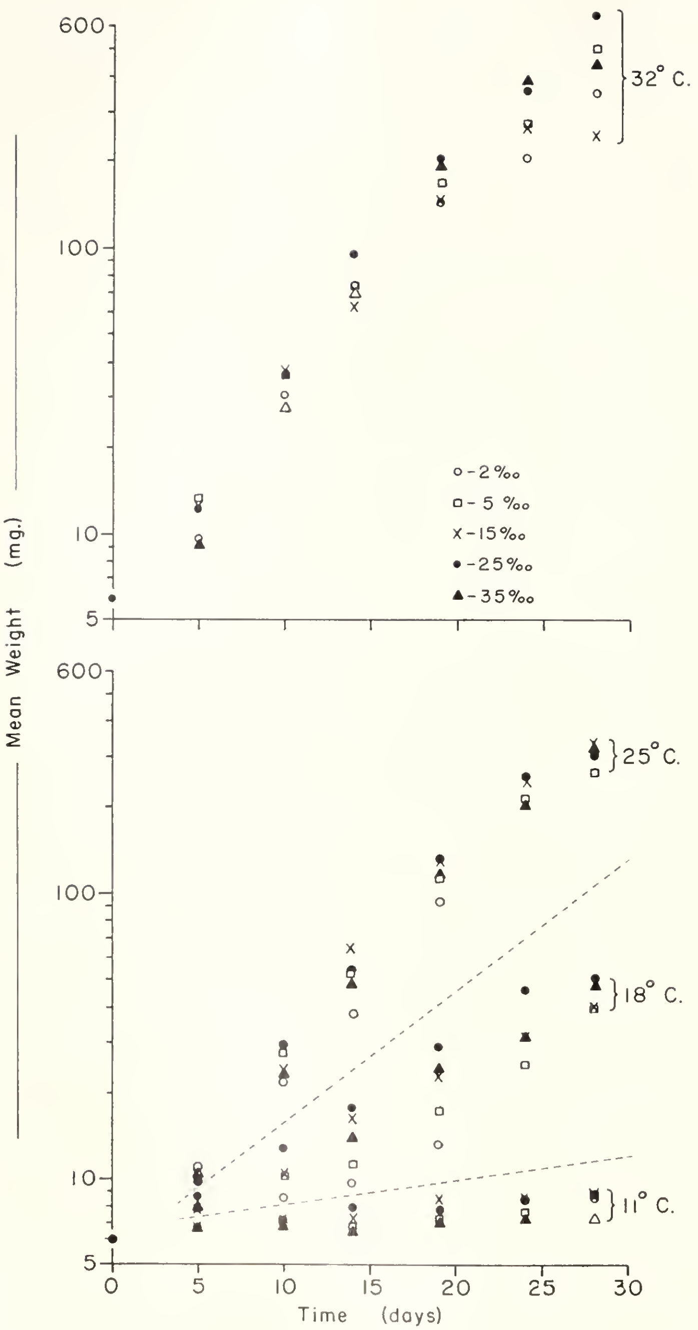

FIGURE 3. Growth of young *P. aztecus* at indicated levels of temperature and salinity.

for those exposed only 24 hours. This apparently paradoxical situation is probably related to the longer acclimation period employed in the 28-day study.

Survival was markedly reduced at the highest temperature (32°C.) at all salinities tested except 35‰. Much of the accountable mortality at this temperature occurred during the first four days (Table III) and was presumably the result of the immediate stress caused by the changes in environment. However, the stress of salinity acclimation would not seem to explain the poorer survival observed at 25‰ (very near the initial salinity) than at 35‰. The relatively large numbers of unobserved deaths occurring at 32° C. and 2‰, 5‰, 15‰ and 25‰ (Table III) suggest two other possible causes of mortality—the experimental temperature *per se*, and increased cannibalism associated with high temperatures (as noted above in Series 1 and 2 of the 24-hour studies). It is possible that at 32° C. the one-month period of exposure in the growth experiment elicited long-term temperature effects which could not be manifested in the relatively short duration of the 24-hour studies.

TABLE IV

Increase in mean length (mm.) of P. aztecus surviving 28 days at indicated levels of temperature and salinity

Temperature (° C.)	Salinity (‰)				
	2	5	15	25	35
32	23.4	28.9	19.0	32.0	25.8
25	—	21.9	24.4	22.3	22.6
18	—	6.3	6.5	7.4	7.6
11	—	—	0.5	0.5	0.4

Mortalities occurring at other temperatures were limited to the lowest salinities, with stress due to reduced salinity and temperature being sufficient to kill all the animals at 2‰ and 11° C. in only 5 days (Table III). The mortalities observed at 5‰ and 11° C., as well as those at 2‰ and 18° C., probably reflect the cumulative effects of stress, since deaths occurred continuously throughout the course of the experiment. At 25° C. and 2‰, however, all observed deaths occurred during a four-day period late in the experiment. Although the initial cause of this mortality is not known, later deaths (on the 21st through the 24th day) were probably due to fouling of water, since brine shrimp were also dying. Furthermore, an earlier experiment (Zein-Eldin, 1963) had indicated that *P. aztecus* postlarvae survive well under these conditions.

Growth

Differences in rate of growth were more closely related to temperature than to salinity (Fig. 3). The relative effects of the two factors may be readily determined by comparing the magnitude of growth differences associated with variation in salinity (columns) with that due to variation in temperature (rows) (Table IV). Differences in mean length between temperature groups were detectable as early as the first sampling period (5 days) and increased in magnitude during the experimental period (Fig. 4). Both length and weight increased much more rapidly at 32° and 25° C. than at lower temperatures. The maximum increase in size was observed at 32° and 25‰, conditions under which one animal grew to 50 mm. and 962 mg., a more than four-fold increase in length and a weight increase of 150-fold (Table V). The great variation in size noted earlier (Zein-Eldin, 1963) was also observed in this experiment, with differences in length between smallest and largest animals ranging from 13 to 25.5 mm. in the various salinities at 25° and 32° C. (Table V). Almost no growth was detected at 11° C., although survival was good at salinities of 15‰ and above. With the exception of 32° C. and corresponding salinity levels of 25‰ and below, where mortality more than offset the rapid growth rate (Tables IV and VI), the gain in total weight of the survivors was comparable within a temperature. However, this gain increased approximately 10-fold within each level of salinity between 11° and 18° C., and only slightly less between 18° and 25° C. (Table VI).

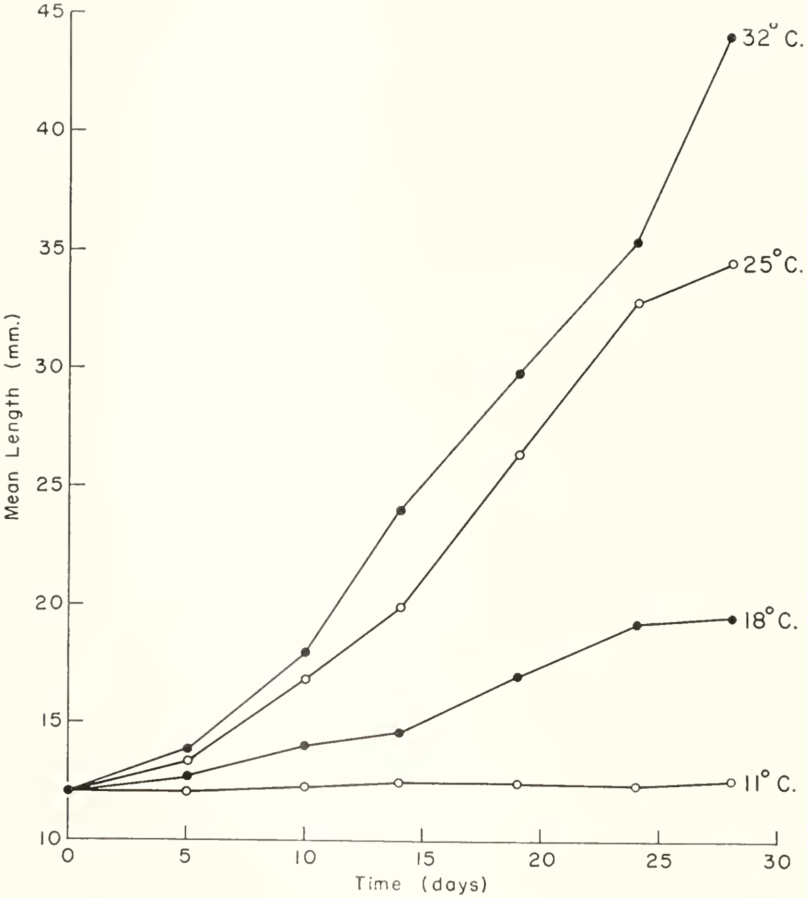

FIGURE 4. Growth of young *P. aztecus* at various temperatures (salinity: 25‰).

TABLE V

Mean size and growth rate of growth-experiment survivors, including 10 animals sampled at 28 days and shown in Table VII. Size range given in parentheses. Initial weight and length were 6.1 mg. and 12.1 mm., respectively

Temperature and salinity	Number of survivors	Weight (mg.)	Length (mm.)	Increase in length per day (mm./day)
32° C.				
2‰	15	340.6 (157.7–735.1)	34.8 (27.5–47.5)	0.81 (0.55–1.26)
5‰	22	447.6 (241.0–667.6)	39.6 (33.0–46.0)	0.98 (0.75–1.21)
15‰	25	240.2 (35.2–542.0)	31.0 (17.0–42.5)	0.68 (0.18–1.09)
25‰	29	610.8 (309.0–961.9)	43.1 (36.0–50.0)	1.11 (0.85–1.35)
35‰	47	423.7 (164.7–753.6)	38.3 (28.5–46.5)	0.94 (0.59–1.23)
25° C.				
5‰	43	274.0 (115.3–482.0)	33.9 (25.0–41.5)	0.77 (0.46–1.05)
15‰	43	375.3 (163.8–681.1)	37.4 (29.0–46.5)	0.90 (0.60–1.23)
25‰	50	313.8 (101.4–538.0)	35.2 (24.0–44.0)	0.82 (0.42–1.14)
35‰	49	291.0 (108.0–605.7)	34.4 (24.5–43.0)	0.80 (0.44–1.10)
18° C.				
5‰	41	33.9 (18.0– 56.2)	18.3 (14.0–21.5)	0.20 (0.08–0.34)
15‰	46	43.7 (15.3– 77.7)	19.1 (14.5–23.0)	0.25 (0.09–0.39)
25‰	48	52.8 (15.0–101.4)	20.1 (14.0–25.0)	0.29 (0.08–0.46)
35‰	49	35.2 (17.7– 62.7)	18.3 (14.5–22.0)	0.22 (0.09–0.35)
11° C.				
15‰	49	8.7 (5.2–13.1)	12.6 (11.0–14.0)	0.03 (0–0.08)
25‰	46	8.8 (6.4–10.8)	12.7 (11.5–14.0)	0.02 (0–0.08)
35‰	43	7.6 (5.5– 9.7)	12.4 (11.0–13.0)	0.01 (0–0.08)

Growth rate based only upon the steepest portions of the growth curves (*i.e.*, between the 10th and 28th days) approached a value of 1.4 mm. per day at 32° C. and 1.1 mm. per day at 25° C., as against the lower values of 1.1 mm. per day at 32° and 0.8 mm. per day at 25° C. over the entire experimental period (Fig. 4, Table VII). Although the mean growth rates reported here (Table V) for both 25° and 32° C. exceed the maximum of 0.56 mm. per day which Pearson (1939) reported for laboratory-held postlarvae of *P. brasiliensis* (probably *P. aztecus*), and the maximum of 1.35 mm. per day far exceeds his value, these rates do not

TABLE VI

Increase in total weight (g.) of P. aztecus surviving 28 days at indicated levels of temperature and salinity. Food conversion efficiency (%) indicated in parentheses where survival was 85% or greater

Temperature (° C.)	Salinity (‰)				
	2	5	15	25	35
32	4.7	9.6	5.8	17.5	19.6 (37)
25	—	11.5 (43)	15.9 (53)	15.4 (46)	14.0 (43)
18	—	1.2 (32)	1.7 (33)	2.2 (40)	1.6 (34)
11	—	—	0.1 (12)	0.1 (14)	0.1 (5)

approach that of postlarval white shrimp, *P. setiferus,* which grew an average of 2.1 mm. per day in pond experiments conducted by Johnson and Fielding (1956). A similarly low rate of growth for aquarium-held *Metapenaeus mastersii,* which ranged in carapace length from 1.9 to 7.0 mm., has been reported by Dall (1958). Laboratory animals grew only 10 mm. in total length per month at 24° to 28° C., as against a natural growth rate of 20 to 30 mm. per month.

Growth of our laboratory-held *P. aztecus* postlarvae likewise only approached that of slightly larger animals in the field. Viosca (1920) estimated a growth rate of approximately 25 mm. per month for *P. setiferus* in the length range 30 to 150 mm., while Gunter (1950) observed a rate of 25 to 40 mm. per month for the same species growing from 28 to 100 mm. Extrapolation of Lindner and Anderson's (1956) growth curve for white shrimp gives a rate of 1.50 mm. per day for shrimp between 20 and 65 mm. in length. Williams (1955) determined a rate of 1.7 mm. per day for *P. aztecus* growing from 37 to 102 mm., a rate also estimated by St. Amant, Corkum and Broom (1963) for 51- to 125-mm. specimens of this species. It must be noted, however, that these estimates were for shrimp at the upper end of the size ranges encountered in the studies described here.

Although the studies of conversion efficiency were necessarily crude, the resulting data indicated that the most efficient utilization of food occurred at 25° C. (Table VI). Because of the high mortality, no efficiencies could be calculated for shrimp held at 32° C. in salinities of 25‰ and lower. The conversion values should be considered maximal at the highest temperatures since some cannibalism probably occurred. However, efficiencies for the animals held at 11° C. are probably low since it was apparent that much of the food provided was not eaten. At this low temperature, the postlarvae were generally inactive, resting most of the time on the bottom.

Johnson and Fielding (1956), studying *P. setiferus* in aquaria during August (temperature not stated), found a mean food-conversion efficiency of 19% for juveniles (mean weight 0.9 to 2.1 g.) held one week at 18.5‰, as against an efficiency of 24% for juveniles (mean weight 0.7 to 2.1 g.) held one week at 34‰. All animals were fed at a rate of 10% of the initial body weight per day, a rate less than that provided in our experiments. The lower efficiencies determined by Johnson and Fielding may be due, in part, to the larger size of the animals held, since it has been shown in fishes (Kinne, 1960) that conversion efficiency decreases with increasing size. Furthermore, Johnson and Fielding obtained the maximum efficiency of 50% from a group of animals of 0.7 g. mean weight held at 34‰. This value compares favorably with those reported here for brown shrimp of mean weight 0.3 g. and less, and would indicate that rapidly growing young shrimp require 2 to 4 g. of utilizable food to produce 1 g. of tissue.

The decrease in growth rate of animals held at 32° C. and 15‰ is unexplainable. This group of animals consumed less food during the latter days of the experiment, even though excess food was present. If this decreased growth represented a long-term effect of the combined stresses of lowered salinity and increased temperature, it is strange that such a decrease did not occur among groups held at even lower salinities at this temperature. Interpretation of the combined effects of lower salinity with 32° C. on growth was complicated by the high rates of mortality among these groups.

TABLE VII

Mean animal weight (mg.) and length (mm.) at each salinity and temperature level. All individuals initially 6.1 (±0.2) mg. and 12.1 (±0.2) mm. Values are based on samples of 10 except those that are starred, which are based on samples of 5. The largest and smallest remaining animals in each test group were included in each sample. Figures in parentheses indicate one standard error

Temp. (° C.) and elapsed time (days)	Level of salinity (‰)									
	2		5		15		25		35	
	Weight	Length	Weight	Length	Weight	Length	Weight	Length	Weight	Length
32°										
5	9.7* (0.9)	13.1* (0.3)	13.5 (1.0)	14.0 (0.4)	12.4 (0.9)	13.8 (0.3)	12.1 (1.0)	13.8 (0.3)	9.3 (0.6)	12.8 (0.3)
10	31.1* (4.9)	17.4* (0.3)	35.9 (3.8)	17.6 (0.6)	37.8 (7.8)	17.8 (1.0)	36.8 (5.8)	18.0 (0.9)	28.1 (4.3)	16.6 (0.7)
14	74.7* (15.8)	22.3* (1.5)	75.8* (12.0)	23.0* (1.2)	62.2 (10.9)	20.8 (1.0)	94.6 (13.0)	24.0 (1.2)	70.2 (9.8)	21.5 (1.0)
19	144.5* (11.2)	27.1* (2.3)	167.3* (16.6)	28.3* (0.9)	148.2* (33.6)	26.6* (2.2)	206.5 (34.7)	29.8 (2.0)	195.8 (23.0)	30.1 (1.2)
24	209.3* (61.0)	30.0* (2.9)	271.3 (32.0)	33.4 (1.2)	265.1* (59.5)	32.2* (2.6)	356.8 (53.7)	35.4 (1.9)	379.6 (51.5)	36.4 (1.6)
28	347.7 (54.1)	35.6 (1.9)	482.3 (23.0)	41.0 (0.6)	246.9 (53.6)	31.1 (2.5)	654.0 (73.8)	44.1 (1.7)	437.2 (57.8)	38.0 (1.8)
25°										
5	9.7 (0.7)	13.1 (0.3)	10.4 (0.7)	13.4 (0.2)	10.8 (0.5)	13.3 (0.2)	11.1 (0.6)	13.4 (0.3)	10.5 (0.6)	13.0 (0.2)
10	21.8 (2.0)	15.9 (0.5)	27.9 (3.0)	17.0 (0.5)	23.0 (3.1)	15.8 (0.6)	29.9 (4.6)	16.8 (0.8)	23.0 (2.2)	16.1 (0.5)
14	37.9 (3.4)	18.4 (0.6)	51.1 (8.6)	20.0 (1.1)	64.7 (12.5)	21.2 (1.3)	54.0 (8.4)	19.9 (1.0)	48.3 (5.1)	19.6 (0.7)
19	93.2 (9.6)	24.0 (1.3)	113.7 (20.6)	24.9 (1.4)	126.7 (22.4)	26.1 (1.3)	132.4 (17.1)	26.4 (1.1)	118.6 (16.9)	25.4 (1.4)
24	—	—	217.0 (25.6)	31.1 (1.2)	245.1 (24.9)	32.4 (1.2)	257.8 (27.0)	32.8 (1.1)	202.7 (27.8)	30.6 (1.2)
28	—	—	264.4 (27.4)	33.0 (1.2)	357.1 (41.8)	36.6 (1.4)	302.0 (41.2)	34.4 (1.7)	321.3 (44.3)	34.8 (1.3)
18°										
5	6.5 (0.2)	12.7 (0.1)	7.5 (0.2)	12.8 (0.1)	8.0 (0.4)	12.8 (0.2)	8.6 (0.5)	12.7 (0.2)	7.9 (0.2)	12.6 (0.1)
10	8.7 (0.4)	12.8 (0.2)	10.0 (0.1)	13.1 (0.1)	10.5 (0.6)	13.2 (0.2)	12.9 (0.8)	14.0 (0.2)	10.4 (0.6)	13.6 (0.3)
14	9.7 (0.5)	13.2 (0.2)	11.4 (0.8)	14.0 (0.3)	16.5 (1.6)	15.0 (0.5)	17.7 (1.6)	14.6 (0.5)	14.0 (1.0)	14.0 (0.3)
19	13.2* (1.1)	14.1* (0.2)	17.4 (1.0)	14.7 (0.2)	23.0 (1.7)	16.0 (0.1)	29.0 (3.6)	17.0 (0.7)	24.2 (2.1)	15.8 (0.3)
24	—	—	25.1 (2.7)	16.1 (0.6)	31.8 (2.9)	17.8 (0.5)	46.0 (4.7)	19.2 (0.6)	31.2 (3.6)	17.6 (0.5)
28	—	—	40.1 (4.0)	18.4 (0.7)	40.6 (4.9)	18.6 (0.7)	49.9 (7.7)	19.5 (1.0)	47.4 (4.0)	19.8 (0.7)
11°										
5	—	—	6.7 (0.3)	12.0 (0.2)	6.7 (0.3)	12.1 (0.1)	6.4 (0.3)	12.1 (0.1)	6.8 (0.2)	12.4 (0.1)
10	—	—	7.1* (0.3)	12.4 (0.2)	7.0 (0.3)	12.0 (0.2)	7.2 (0.3)	12.2 (0.2)	6.9 (0.4)	12.3 (0.2)
14	—	—	6.9* (0.3)	12.3 (0.1)	7.3 (0.2)	12.5 (0.2)	8.0 (0.3)	12.5 (0.2)	6.5 (0.3)	11.9 (0.2)
19	—	—	7.3* (0.4)	12.4 (0.1)	8.6 (0.3)	13.0 (0.2)	7.8 (0.3)	12.4 (0.2)	7.2 (0.3)	12.3 (0.1)
24	—	—	7.7* (0.3)	12.6 (0.1)	8.7 (0.6)	12.6 (0.2)	8.3 (0.4)	12.4 (0.2)	7.2 (0.4)	12.4 (0.3)
28	—	—	—	—	8.7 (0.4)	12.6 (0.2)	8.6 (0.4)	12.6 (0.2)	7.3 (0.4)	12.5 (0.2)

Biological and ecological implications

Commercially important North American shrimp of the genus *Penaeus* spawn at sea. As shown for *P. setiferus*, the larvae develop in the open sea, migrate into the estuarine areas as postlarvae, remain in the less saline estuaries until they approach maturity, and then return to the sea (Weymouth, Lindner and Anderson, 1933; Burkenroad, 1934; Pearson, 1939). Various studies in the field have suggested that postlarval and juvenile *Penaeus* are associated with low salinities characteristic of the estuary, and that postlarvae require the lowest salinity for growth and survival (Gunter, 1945, 1950; Pearse and Gunter, 1957). Lindner and Anderson (1956) concluded, however, that size of white shrimp (juveniles and subadults) seemed more closely related to locale than to salinity. Gunter, Christmas and Killebrew (1964) have recently presented additional field data indicating differences in the natural distributions, with respect to salinity, of the three commercial species, *P. aztecus*, *P. duorarum*, and *P. setiferus*. In so doing, these authors have made certain assumptions. For example (p. 184): "If salinity meant nothing to these animals they would be evenly distributed relatively over the whole range, if food were available. The general food habits of shrimp are still largely unknown, but all indications are they are omnivorous feeders, and shrimp do find food over the full salinity range up to pure sea water, although the food doubtless changes with size." The fact that shrimp do apparently eat a variety of food does not, however, indicate that all such foods are of comparable nutritive value (Williams, 1959; Zein-Eldin, 1963). Furthermore, there is no evidence that food is equally available throughout the salinity range occupied by shrimp in nature. Only in a previous study (Zein-Eldin, 1963) and in the work reported here, has food been equally available to all animals regardless of salinity. The 24-hour survival experiments, as well as the growth study, indicated that for *P. aztecus* postlarvae, only extreme salinity conditions influence growth and survival. Even normal oceanic salinity is not sufficient to interfere with postlarval brown shrimp growth and survival when other factors (temperature, food supply, predation, oxygen, light, pollution, etc.) are kept relatively constant. In view of our results, we suggest that other factors, such as food or cover (which may themselves require relatively narrow salinity ranges), are of greater importance than salinity *per se* in determining distribution, growth, and survival of these animals.

In the present studies, both the survival and the growth data indicated that wide ranges of salinity and temperature were well tolerated by postlarval brown shrimp. The combination of low salinity and low temperature, however, was not favorable, either for survival or growth. That *P. aztecus* can withstand extreme conditions of both factors has been demonstrated in the field as well, although published records have been largely limited to occurrences of juvenile and adult forms (Gunter, 1950). Bearden (1961), who found postlarval brown shrimp at temperatures as low as 6.5° C., noted a marked decrease in their abundance following the sudden cold spell which resulted in this low-temperature value. Renfro and Baxter (unpublished) have reported live postlarvae at 12° C. and 31.0‰ as well as at 2° C. and 30.5‰, supporting our laboratory evidence that low temperatures can be survived when salinities are sufficiently high. Comparable data of postlarval occurrence in low-salinity areas are not yet available. Brown shrimp (size not stated) have also been reported in salinities as low as 0.8‰

(Gunter and Shell, 1958) in Louisiana, while Gunter and Hall (1963) report a 34- and a 38-mm. specimen at 0.22‰ in the St. Lucie estuary in Florida. No temperatures were reported with the latter data, however. It must be noted, nevertheless, that St. Amant, Corkum and Broom (1963) reported maximal spring abundance of postlarval brown shrimp in Louisiana bays only after water temperatures consistently exceeded 20° C.

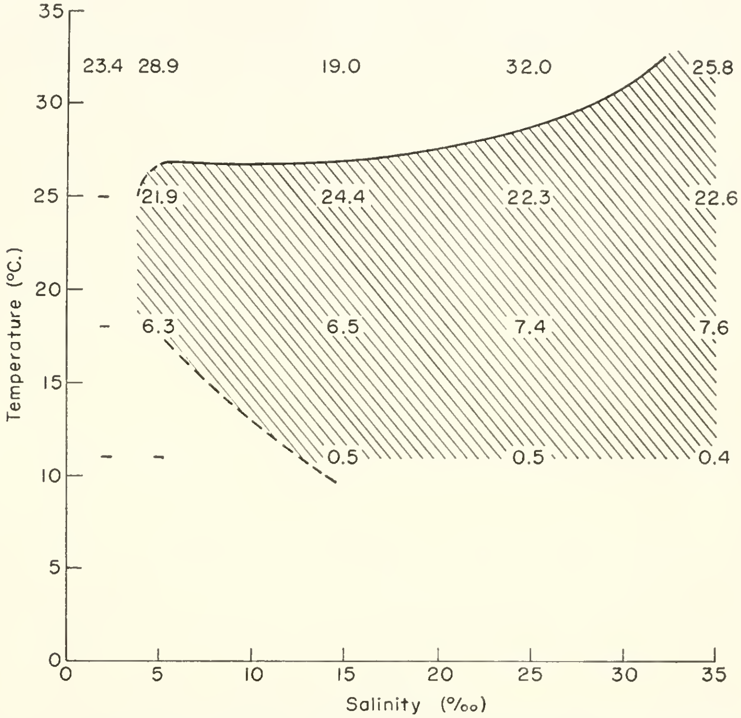

FIGURE 5. Growth and survival of young *P. aztecus* held 28 days at indicated levels of temperature and salinity. Numbers indicate increase in mean length (mm.). Hatched zone indicates 80–100% survival.

The temperature range permitting growth is more limited than the range for survival (Fig. 5). Our laboratory studies have demonstrated that growth can occur over a wide range of salinity at temperatures of 25° C. and above, and suggest that the effect of temperature upon the rate of growth increases rapidly with temperature between 11° and 25° C. (Fig. 6). This effect of temperature has been confirmed in more recent experiments in which we observed growth at a greater number of temperature levels between 15° and 35° C. than tested here.

The greatest growth differential per 7° C. was observed between the 18° and 25° C. levels. This difference may well explain the observation of St. Amant, Corkum and Broom (1963, p. 25) that "metamorphosis of postlarvae into rapidly growing juveniles occurs suddenly after water temperature exceeds 20° C." Above 25° C., increasing temperature has less effect upon growth. The recent experiments referred to above indicate that growth is maximal at 30° to 32.5° C. This result, coupled with the increased mortality at 32° C., suggests that such a temperature condition, although promoting rapid growth in some individuals, may be above the optimum temperature for long-term growth and survival of *P. aztecus* postlarvae.

The laboratory evidence suggests that normal winter temperatures render the brackish bay systems unfavorable for both survival and growth of brown shrimp postlarvae, whereas almost any salinity will provide a favorable environment at normal summer temperatures. Thus, the pattern of tolerance to salinity and temperature observed in the laboratory may explain the seasonal distribution of *P. aztecus* in much the same manner as described by Broekema (1941) for the migratory European shrimp, *Crangon crangon*. Survival of postlarvae within the estuary may also be affected by decreases in temperature or salinity. In the spring, postlarvae entering bays having relatively low temperatures and salinities above 15‰ may be adversely affected by a sudden salinity drop, such as that caused by heavy spring rains. Conversely, if the temperatures are intermediate (18° C., for example) but salinities low (10‰ or less), a drop in temperature may also decrease survival. Simultaneous decreases in both physical factors—temperature and salinity—would be most detrimental to the population in terms of both survival and growth.

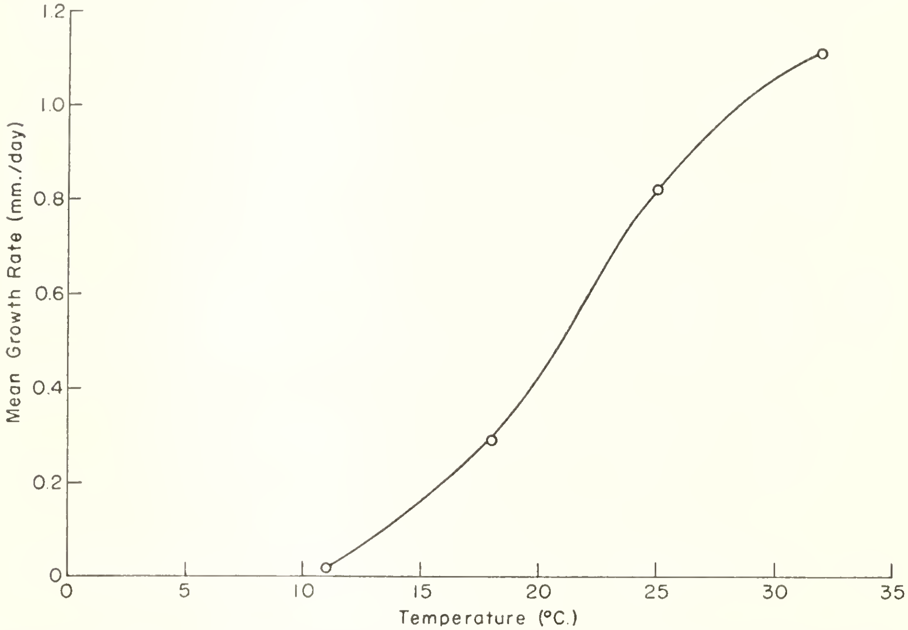

FIGURE 6. Effect of temperature on the laboratory growth rate of young *P. aztecus* (salinity: 25‰; length of experiment: 28 days; initial length of experimental shrimp: 12.1 mm.).

Williams (1960) had previously noted the effects of temperature and salinity on juveniles and subadults of *P. aztecus*. Not only did he find that the 96-hour survival of 42- to 100-mm. specimens declined with decreasing temperature over the range 28.8° C. to 8.8° C., but he also determined that survival was most markedly reduced at 10‰ (the lowest salinity tested) regardless of the temperature. Animals exposed to 8.8° C. showed a greater tendency to lose the ability to regulate the osmotic concentration of the serum. It is of interest that juveniles were better able to regulate serum concentration than were adults (120 to 150 mm.) exposed to the same conditions. McFarland and Lee (1963) demonstrated that brown shrimp adults were better osmoregulators at higher salinity than at lower, with a greater tendency to isosmoticity when the external medium was below 18‰. The latter authors were unable to study animals in salinities below 5‰ to 6‰ since only one of 12 adults survived 24-hour exposure to this range of salinity, despite an acclimation period of almost one week.

The studies cited above suggest that salinity tolerance may vary not only with temperature, but also with size (age) of the shrimp. In demonstrating good survival of *P. aztecus* postlarvae over a broad range of salinity and temperature, the findings presented here suggest that postlarvae of this species are better osmoregulators than juveniles, which were tested by Williams (1960), or the adults tested by McFarland and Lee (1963). Further studies are planned to determine the effects of both temperature and salinity upon the osmotic behavior in various life-history stages of *P. aztecus* and *P. setiferus*.

SUMMARY

1. The combined effects of salinity and temperature upon growth and survival of postlarvae of the brown shrimp, *Penaeus aztecus*, were studied under controlled conditions.

2. Test salinity ranged from 2‰ to 40‰ and temperature from 7° to 35° C.

3. With relatively short periods of acclimation, postlarval brown shrimp withstood wide fluctuations in both temperature and salinity for 24 hours.

4. The range of tolerance to these factors over periods of 28 days was only slightly less than that observed for 24 hours.

5. Postlarvae survived temperatures as low as 11° C. with almost no growth for one month in salinities of about 15‰ or above.

6. Growth increased with temperature, with significant growth beginning at some temperature above 11° C. but below 18° C. The most marked increase in growth rate occurred in the temperature region between 11° and 25° C.

7. At temperatures below 15° C., young (postlarval) shrimp demonstrated a decreased tolerance to low salinity. This reduced tolerance may influence the natural distribution and survival of postlarvae, which do not ordinarily enter the estuaries in abundance until spring when the temperature has increased to levels at which characteristically low estuarine salinities are no longer harmful.

8. Salinity *per se* had little effect on either survival or growth, except at extreme temperatures.

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TUBE-BUILDING AND FEEDING IN CHAETOPTERID POLYCHAETES

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The tube-building and feeding behavior of the large familiar *Chaetopterus variopedatus* has been known since the studies of Enders (1908, 1909) and MacGinitie (1939). Much less was known about the other members of the family Chaetopteridae until 1964, when the Atlantic chaetopterid, *Spiochaetopterus oculatus*, was investigated by the author. This little species, having a pair of long palps and inhabiting a straight vertical tube, is a much more typical member of the family than is *Chaetopterus*. The purpose of this study therefore was to investigate the tube-building and feeding mechanisms of the remaining four chaetopterid genera; *Telepsavus, Phyllochaetopterus, Ranzanides,* and *Mesochaetopterus.*

The greater part of this study was carried out in Naples, Italy, where representatives of every chaetopterid genus except *Mesochaetopterus*, occur in the Bay of Naples. The author wishes to express his appreciation to the Stazione Zoologica at Napoli for the facilities and courtesies extended to him during the three months of residence at the laboratory, and also to the National Science Foundation for their support of space utilized at the laboratory. The author is indebted to Dr. R. Phillips Dales of Bedford College, University of London, who provided unpublished data from his observations of *Mesochaetopterus,* and also to Dr. Marion Pettibone of the U. S. National Museum for the loan of specimens of *Mesochaetopterus.*

MATERIALS AND METHODS

All of the living specimens utilized in this study were collected by dredging in water ranging in depth from two to 50 meters. The small size and relatively shallow depth of the tubes of these chaetopterids prevented any excessive damage by the dredge.

To study feeding and other behavior the worms were transferred from their natural tubes into glass capillary tubes. The diameter of the capillary tube was critical for the adaptation of the worms to this artificial environment; but if the fit was a good one, the worms lived for as long as two months. The process of transfer from the natural tube to a glass capillary tube was most easily accomplished

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by means of a hypodermic syringe with a small needle. The needle was inserted into one end of the natural tube and a strong stream of sea water was then injected. This rapidly forced the worm in an undamaged condition out of the opposite end of the tube. The needle of the syringe was then inserted into one end of the glass capillary tube and the syringe slowly filled. The filling syringe created a current of sea water through the tube sufficient to suck the worm into the capillary tube.

The capillary tubes were placed within a glass cylinder. A plug of glass wool held the tubes in a vertical position against the inner wall of the cylinder, and a short piece of large glass tubing penetrated the center of the plug to permit adequate water circulation. When the worms were not being studied, the entire glass cylinder was submerged within a tank of circulating sea water. The worms were observed through the cylinder wall by means of a horizontally oriented dissecting microscope. The base of the microscope had been removed and the upper optical portion was attached in a horizontal position to a ring stand.

Observations of tube-building were facilitated by confining the worms to very short sections of natural tube. The worms would then frequently construct additions to the tube. Determination of water current and degree of tube obstruction was aided by the use of carmine-stained sea water injected into the tube. A suspension of carmine particles in sea water was used to study ciliary tracts and feeding. Observations of feeding were also aided by using an artificially stained detritus. The detritus was prepared by boiling a small amount of cooked pasta in carmine and then grinding and suspending it in sea water.

RESULTS

Telepsavus costarum Claparède

Telepsavus costarum Claparède is one of two species known for the genus. It is cosmopolitan and in the Western Hemisphere occurs along the Pacific coast of North America. This species was collected from the Bay of Naples at depths of four to five meters from a bottom of fine sand and silt.

The tube of *Telepsavus* and the structure of the worm itself are almost identical to that of *Spiochaetopterus* (Fig. 1, B). *Telepsavus* secretes an opaque cornified annulated tube (Figs. 2 and 3, A) which is buried vertically in the substratum. Only a small part of one end of the tube projects above the surface of the sand. In the Bay of Naples the longest dredged tube was 25 cm. but longer tubes are probably common. The internal diameter ranged from 1.2 to 1.4 mm. The lower region of the tube commonly contains a secreted button-like transverse partition (Fig. 3, E) which is perforated by several openings to allow a water current to pass through the tube.

The total length of *Telepsavus costarum* averages about 8 cm. and, as in all chaetopterids, the body is divided into three regions. The anterior region (Fig. 1, A) contains nine segments, indicated externally only by the presence of the nine short lobe-like notopodia; the anterior neuropodia are lost in all chaetopterids. Each notopodium is supplied by a bundle of capillary setae. The fourth notopodium, in addition, carries a large, heavy, blade-like seta. The anterior end of the body (Fig. 3, C) is truncate, the ventral margin projecting beyond the mouth.

A small lip flanks the mouth dorsally and lies between the bases of two long heavy palps, which may equal or exceed half the body length.

The entire convex ventral surface of the anterior body region is covered by a thick glandular epidermis which secretes the tube. The tube is secreted in half-cylinder sections. When an addition to the tube is to be secreted, the worm slowly extends almost the entire anterior region of the body out of the tube. Simultaneously, the body is flexed dorsally at the level of the 4th or 5th segment and appears very flared and turgid. At the end of the extension movement, the body is quickly withdrawn into the tube, leaving behind a delicate half-cylinder of new tube. The worm then rotates 180° within the tube and secretes the opposite half-cylinder. The length of the addition corresponds to the distance between two annulations.

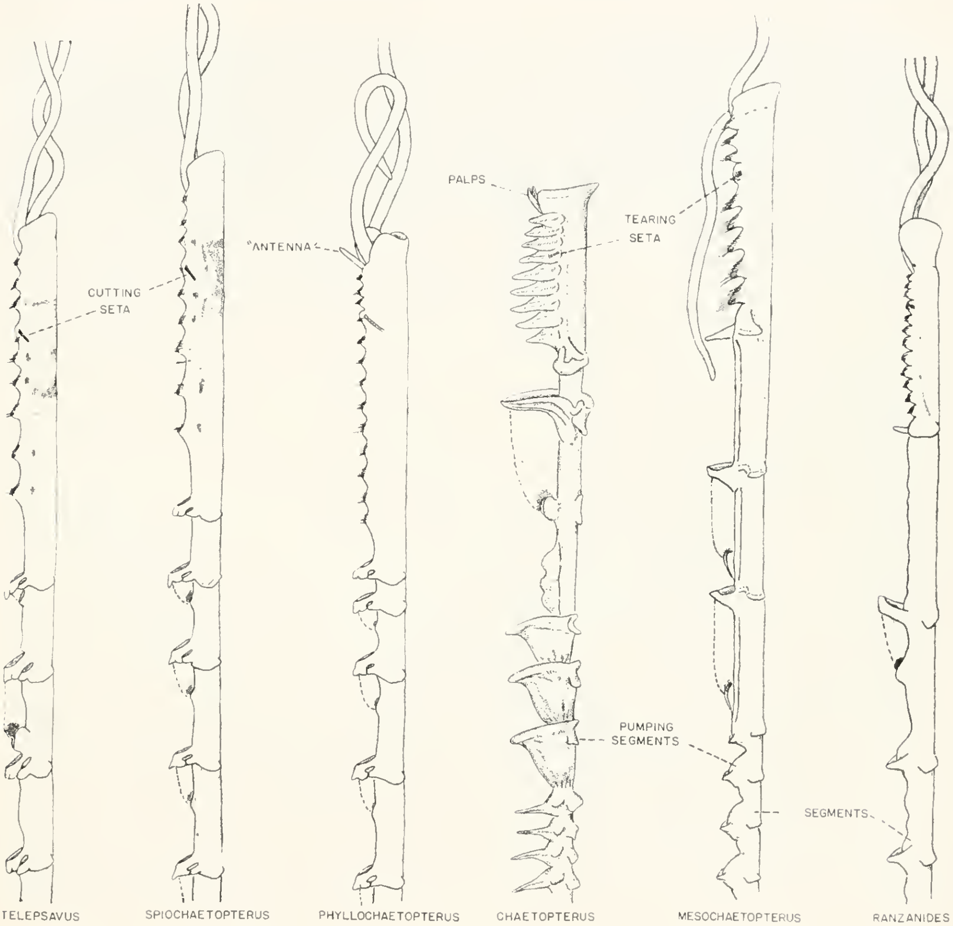
PALPS
TEARING SETA
ANTENNA
CUTTING SETA
PUMPING SEGMENTS
SEGMENTS
TELEPSAVUS
SPIOCHAETOPTERUS
PHYLLOCHAETOPTERUS
CHAETOPTERUS
MESOCHAETOPTERUS
RANZANIDES

FIGURE 1. Lateral view of anterior and middle body regions of representatives of each of the six chaetopterid genera. Mucous bags are indicated by shaded areas enclosed by dashed lines.

The laying down of a transverse partition was never observed in *Telepsavus.* However, since the partitions are identical to those of *Spiochaetopterus,* they are probably constructed in the same manner (Barnes, 1964). In *Spiochaetopterus* the worm assumes a head-downward position in the tube and at the level at which a partition is to be placed, the head of the worm is slightly flexed dorsally. The partition is then laid down during a rapid rotating movement of the anterior end. The anterior ventral margin of the head projects through a central opening in the partition at the end of the rotation. This single opening is later reduced to two to four smaller openings as the secretory surface of the worm is moved back and forth across the partition. Why the opening is not completely obliterated is not understood.

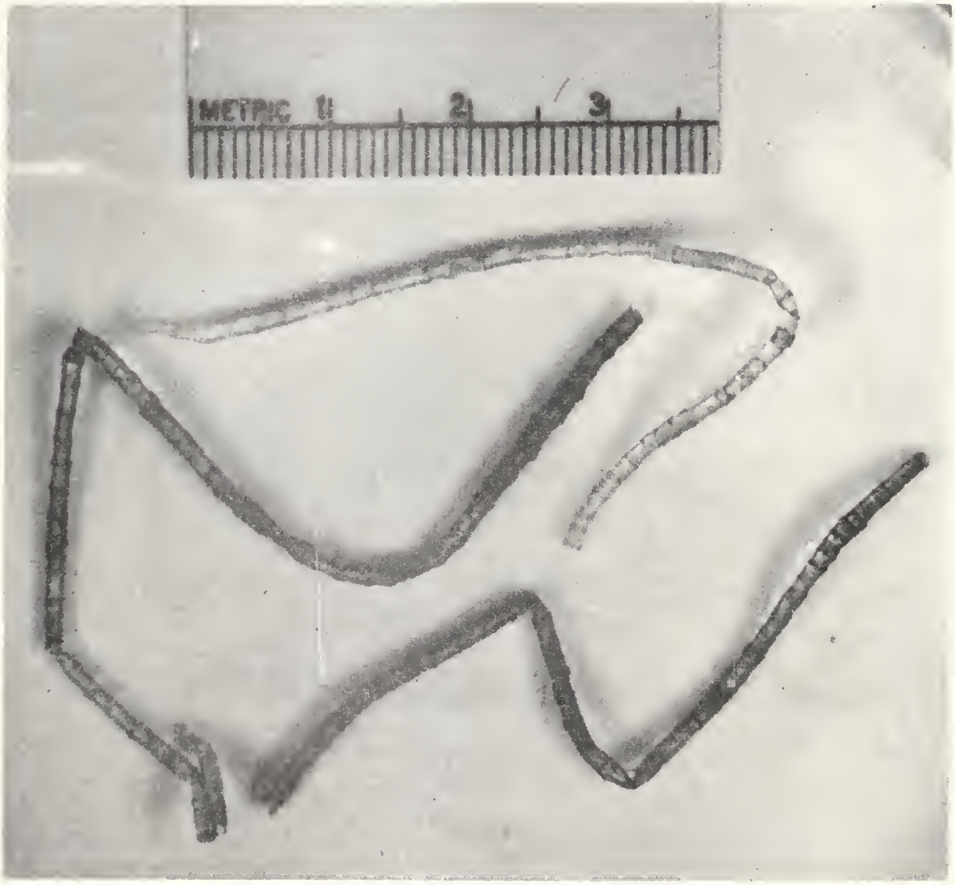

FIGURE 2. Part of the tube of *Telepsavus costarum.*

Partitions are removed exactly as in *Spiochaetopterus.* The worm assumes a head-downward position in the tube. At the level of the partition the body is flexed dorsally 180° so that the angle of flexure is located at the 4th parapodia. The flexed region of the body is now rotated a little to one side, usually toward the left side, so that the enlarged blade-like 4th seta is directed downward against the periphery of the partition. The seta is now extended, cutting through at the junction of the partition and the wall. Then the seta is retracted. Following each stroke, the worm rotates slightly in the tube. In less than two minutes the worm neatly cuts the partition completely free. The old partition is pushed downward and against the tube wall and is gradually incorporated into the wall itself as additional reinforcing secretions are laid down by the worm against the inner surface of the tube.

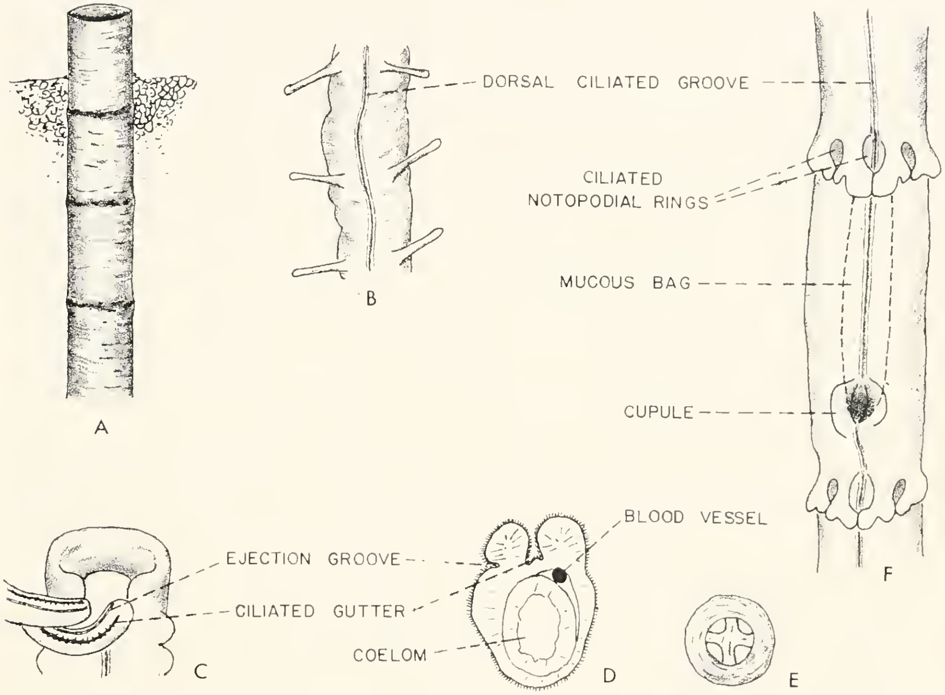

Figure 3. *Telepsavus costarum.* A. Upper end of tube. B. Dorsal view of three segments of posterior body region. C. Dorsal view of anterior end of body showing grooves on palps. D. Cross-section of palp. E. Surface view of a tube partition.

The modified 4th setae are also used to cut open or rupture the tube wall, permitting the formation of a new extent of tube at this point. Such a new addition was always observed at the lower end of the tube and the entrance to the old lower section of the tube was sealed over.

The middle body region commonly contains 31 segments although the number is not constant. The parapodia are biramous. The neuropodia (Fig. 1, A) are broad, rounded lobes provided with uncinate setae and are used for anchoring the body to the tube wall. The notopodia have a foliaceous structure (Fig. 3, F). Each notopodium consists of two main divisions, one dorso-medial and one dorso-lateral. The dorso-medial division is again divided into two rami. One ramus is directed dorso-medially and contacts the corresponding ramus of the opposite notopodium. Together the two opposing rami enclose a large ring-like mid-dorsal opening. The other ramus of the dorso-medial division is directed ventrally and contacts the dorso-lateral division of the notopodium. This contact likewise encloses a lateral ring-like opening, one on each side. Thus there are three notopodial rings, one dorsal and two lateral, at the level of each segment in the middle body region.

The posterior body region (Fig. 3, B) varies from a few to many segments. The neuropodia of this region are similar to those of the middle body region; the notopodia are simple antenna-like projections bearing a seta at the tip.

The notopodial rings of the middle body region are lined by large membranelles. The membranelles beat continually and the beating occurs as several counterclockwise waves moving around the ring margin. The beating of the membranelles in the notopodial rings of the middle body region drives water through the tube, creating the water current upon which the worm depends for respiration, for food, and for the elimination of waste.

Like other chaetopterids, *Telepsavus* obtains food by straining the tubal water current through a mucous bag. A single mucous bag is employed by *Telepsavus costarum*. The bag is secreted by the second mid-dorsal notopodial ring (Figs. 1, A and 3, F) which is slightly heavier than the other mid-dorsal rings of the middle body region. The end of the mucous bag is caught by a large ciliated cupule located medially just in front of the third dorsal notopodial ring. The cilia in the cupule beat backward, and roll the gathered end of the mucous bag into a ball. As one end of the bag is rolled up in the cupule, additional mucous film is secreted at the opposite end.

The notopodial ring which secretes the mucous bag is also lined by membranelles and all of the water driven through this ring must pass into and through the mucous bag. Plankton and fine detritus are strained out and incorporated into the slowly enlarging mucous food ball being formed in the cupule. When the food ball has reached a certain size, secretion of mucus is halted, and the bag is detached from the notopodial ring. Now the food ball moves out of the cupule and is carried forward along a mid-dorsal ciliated groove (Fig. 3, F). The flanking ridges of the groove bifurcate just in front of the small dorsal lip of the mouth. One ridge of the groove passes toward the side of the lip, and the other ridge passes to the opposite side. Each ridge gradually diminishes. The food ball, on reaching the bifurcation of the ciliated groove, passes over the dorsal lip and into the mouth.

A small amount of mucus is secreted by the first notopodial ring and is collected by a rudimentary cupule located immediately behind the ring. The mucus is never elaborated as a distinct bag and appears to be of little importance in feeding.

As in *Spiochaetopterus,* the two long anterior palps play a minor role in feeding. Each palp is provided with a deep ciliated gutter located on the dorsal side (Fig. 3, C and D). Small particles which become lodged in the gutter are carried down the length of the palp by the beating cilia. At the base of the palp the gutter passes onto the lateral margins of the mouth. But only rarely in *Telepsavus* were particles ever observed being carried within the palpal gutter. This was true not only for introduced suspensions of carmine particles and stained detritus but also for natural particles.

The principal function of the palps is the removal of feces and the maintenance of an unobstructed tube. Just medial to the ciliated gutter is a small ciliated groove

(Figs. 3, C and D). In contrast to the downward beating cilia of the larger palpal gutter, the cilia of the smaller groove beat distally. Any undesirable particles which are brought into the tube by the water current must pass over the palps before reaching the body proper and the notopodial rings. When such particles contact the palps they are quickly swept onto the ejection groove and then transported along the groove to the tip of the palp. At the same time the worm moves upward in the tube until the palps project out of the tube opening. Material being carried along the ejection groove drops from the tip of the palps and falls outside of the tube.

The palps are highly effective in clearing the tube of undesirable material. When stained detritus or a suspension of carmine particles was introduced into the upper end of the tube, over 90% was quickly ejected by the palps.

Feces are egested from the posterior anal opening in the form of small pellets. The pellets are immediately picked up by the mid-dorsal ciliated groove, which runs the entire length of the body and in the middle and anterior body regions also functions as the food groove already described. The fecal pellet is carried anteriorly along the groove. In the middle body region the apposing notopodia forming the mid-dorsal ring separate when the pellet moves through the ring. Their separation thus reduces the chance of the pellet being swept out of the groove by the opposing beat of the membranelles lining the ring.

When the fecal pellet reaches the anterior end of the mid-dorsal groove, it does not pass over the dorsal lip as does the food ball. Rather, the fecal pellet follows either one of the two ridges which separate and swing to either side of the dorsal lip. The ridge carries the pellet toward the ejection groove at the base of the palp. The fecal pellet is then carried the length of the palp to the tip. Simultaneously, the worm moves upward in the tube and projects the palps to the outside so that the fecal pellet falls away from the opening of the tube.

Phyllochaetopterus socialis Claparède

Phyllochaetopterus socialis Claparède was dredged from a bottom of fine sand mixed with silt in about two meters of water. Only small numbers of specimens were collected.

This species is a very small chaetopterid, measuring only 15–25 mm. in length not including the palps. The structure of *Phyllochaetopterus socialis* (Fig. 1, C) is essentially like that of species of *Telepsavus* and *Spiochaetopterus.* It differs from the members of the other two genera in only minor respects. In *Phyllochaetopterus* the dorsal lip is very large and the ventral lip is cleft (Fig. 4, D and E). There is a pair of small antennae-like structures located directly behind the palps. They extend anteriorly only slightly beyond the margin of the head. These antennae-like processes occur only in this genus and represent modified parapodia, for each contains a single seta. The true palps of *Phyllochaetopterus* are somewhat shorter than those of *Spiochaetopterus* and *Telepsavus,* approximately equaling the anterior body region in length. The anterior body region contains 13 setigerous segments, and the middle body region contains from four to ten segments.

The tube of *Phyllochaetopterus socialis* tends to be branched and crooked (Fig. 4, A and C). Although part of the tube is always buried, a considerable extent may lie horizontally above the surface of the substratum. The length of the tube ranges from 3.0 to 6.0 cm. and the internal diameter from 0.45 to 0.80 mm. The tube is brown in color and has a tough leathery texture and appearance without any external annulations.

In *Phyllochaetopterus socialis* one to four worms may inhabit a single tube. The individuals may occupy separate branches or the same section of the tube, but there is no definite correlation between the number of tube branches and the number of occupants. In general there is usually one main extent of tube with two openings and any side branches tend to be sealed off from the main section. Multiple occupancy of a single tube is not limited to *Phyllochaetopterus socialis* but occurs in some other members of the genus, such as *Phyllochaetopterus prolifica* from the Pacific coast of North America. The condition is believed to result from fission of the original builder of the tube.

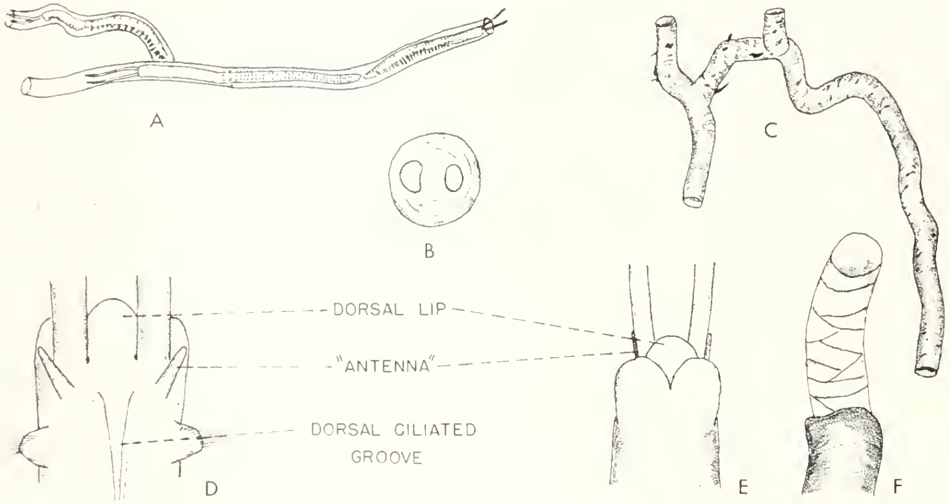

FIGURE 4. *Phyllochaetopterus socialis*. A. Three worms within a common tube. B. Surface view of a partition. C. Part of tube. D. Dorsal view of anterior end of body. E. Ventral view of anterior end of body. F. A recently constructed section of tube adjoining an older section.

Partitions are located within the interior of the tube and occur anywhere along the tube length. The structure of the partition (Fig. 4, B) is similar to those of *Telepsavus* and *Spiochaetopterus*. A number of times two worms were placed in one tube. In one instance one of the worms placed a partition between the two occupants. In other cases no partition was constructed and the two worms frequently passed each other in the tube.

Phyllochaetopterus secretes its tube in a much less regular fashion than does *Telepsavus* or *Spiochaetopterus*. The worm lays down small crescent-shaped overlapping sections of varying size (Fig. 4, F). During the process of secretion, the body of the worm is not markedly arched nor projected very far out of the tube. Partitions are cut out from the tube in the same manner as in *Telepsavus* and

Spiochaetopterus and are probably laid down in the same way also, but they were never observed being secreted.

As in *Telepsavus* and *Spiochaetopterus,* water is driven through the tube of *Phyllochaetopterus* by the beating of the membranelles bordering the rings formed by the foliaceous notopodia of the middle body region.

Phyllochaetopterus utilizes three methods to obtain food. Feeding may involve mucous bags as in *Spiochaetopterus.* A mucous bag is secreted by the middle ciliary ring of the more anterior foliaceous parapodia, except for the first (Fig. 1, C). Each bag is caught by a cupule located behind the ciliary ring. As many as eight mucous bags and rotating food balls were observed being formed at one time.

An alternate method to the use of mucous bags appears to be stimulated by the presence of a heavy concentration of food particles in the water passing through the tube. Under these conditions the notopodial rings spin out a mucous rope instead of bags. The rope picks up and traps particles in the swirling water current streaming through the notopodial rings. The rope joins with that of other segments to form a continuous twisting strand. Mucus for the rope appears to be supplied not only by the notopodial rings which secrete the mucous bags but also by the lateral margins of the mid-dorsal longitudinal ciliated groove. A conspicuous whitish glandular strip flanks the groove in both the middle and posterior body region. The mucous rope extends well into the posterior body region and it may well be partly secreted in this region.

Great quantities of mucus are evident when *Phyllochaetopterus* is removed from its tube. The worm becomes so wrapped up in mucus that it is difficult to handle. The source of the mucus may be the glandular strips bordering the dorsal ciliated groove. Such large amounts of mucus were not found in any other of the chaetopterids studied and none possess the glandular strips.

The palps also seem to be of some importance in feeding. When large amounts of artificial detritus or suspended carmine particles were introduced into the glass tube, the greater part of this material would be collected by the major groove, or ciliated gutter, of the palps and conducted downward to the mouth. Material appeared to be conveyed as easily when the contact was superficial as when material was lodged deeply in the groove.

Feces are removed by way of the dorsal ciliated groove and the ejection groove of the palps. Fecal pellets of specimens in capillary tubes were commonly stuck to the rim of the tube opening or even to the underside of a tube partition, where a partition had been placed above the worm. The palps of *Phyllochaetopterus* are also used to remove undesirable objects from the tube brought in by the water current. But this species is less active than other chaetopterids studied and moves rather slowly up and down the tube.

Ranzanides sagittaria (Claparède)

Ranzanides sagittaria (Claparède), which is known only from the Mediterranean, is the most abundant chaetopterid in the Bay of Naples. Individuals tend to occur together in close associations, and large masses of hundreds of adjacent tubes were dredged from fine silt and sand in 10–12 meters of water. The tubes are composed of an outer layer of sand grains (Fig. 5, A) separated by an inner secreted cornified organic lining, probably of similar composition to that of other chaetopterid tubes. The length of the tubes ranges from 4.5 to 8.5 cm., with a bore of 0.7–1.0 mm. Although these worms occur in compact masses, there is little fusion of adjacent tubes. The tubes are more or less straight, oriented paral-

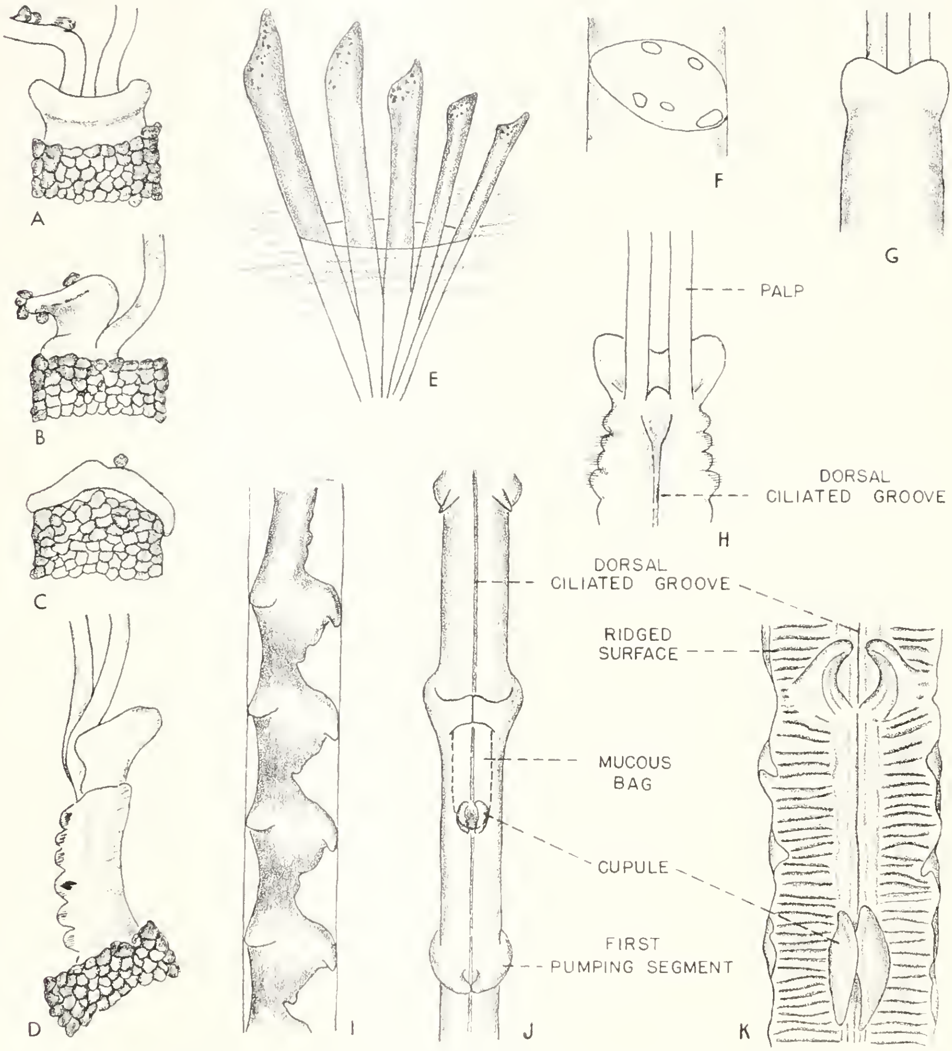
A
B
C
D
E
F
G
PALP
DORSAL CILIATED GROOVE
H
DORSAL CILIATED GROOVE
RIDGED SURFACE
MUCOUS BAG
CUPULE
FIRST PUMPING SEGMENT
I
J
K

FIGURE 5. *Ranzanides sagittaria.* A–D. Tube construction. A. Sand grains being brought to ventral lip by palps. B. Sand grains being moved to under (outer) surface of lip. C. Ventral lip pressing sand grains to rim of tube. D. Worm laying down secreted lining of tube. E. Bundle of enlarged 4th notopodial setae puncturing a tube partition. F. Tube partition in place with five perforations. G. Ventral view of anterior end of body. H. Dorsal view of anterior end of body. I. Lateral view of four middle body segments in pumping position. J. Dorsal view of section of middle body region involved in secreting mucous bag. *Mesochaetopterus taylori.* K. Dorsal view of one segment of middle body region involved in secreting mucous bag.

lel to each other, and rest vertically in the substratum with one opening of the tube at the surface.

Ranzanides is only a little larger than *Phyllochaetopterus socialis*. The length exclusive of the palps, is approximately 2.0 cm. to 2.5 cm. The structure of this species (Fig. 1, F) departs considerably from that found in *Spiochaetopterus, Telepsavus,* and *Phyllochaetopterus*. The palps are long and are similar to those of other chaetopterids. The ventral lip (Fig. 5, G and H) is very large and flaring. Eye spots are present. The anterior body region consists of 12 setigerous segments. The usual single lobate notopodium composes the parapodia. The fourth parapodium carries five very heavy setae in addition to a bundle of ordinary setae.

The middle and posterior body regions (Figs. 1, F and 5, I and J) are not sharply demarcated. All of the parapodia are biramous with the neuropodium uncinate. The first parapodia of the middle body region lie immediately behind the 12th and last parapodia of the anterior body region and possess a short antenna-like notopodium. The second parapodia of this region lie considerably behind the first and their notopodia are large and wing-like. The third and remaining parapodia are placed at regular intervals and the notopodia have the form of short stubby fingers. The length of the notopodia gradually increases posteriorly and the notopodia of the posterior body region eventually assume an antenna-like form similar to that of other chaetopterids.

The secreted part of the tube is laid down by the ventral surface of the anterior body region as in other chaetopterids. The large flaring ventral lip is responsible for molding the outer sand grain layer. In the construction of an addition to the tube, the outer sand grain layer is added to the old tube before the inner secreted lining. This process begins with the collection of sand grains. The anterior end of the worm is projected from the aperture of the tube and may be arched ventrally so that the upper (inner) surface of the ventral lip contacts the substratum. Particles of sand adhere to the mucus on the lip surface. Sand particles may also be collected by the palps (Fig. 5, A) in the same manner. Adhering particles are then conveyed downward to the ventral lip in the major groove of the palp. Not infrequently a palp is wiped against the lip. The lip surface is strongly ciliated and the sand particles are driven over the edge onto the under (outer) surface (Fig. 5, B). Periodically the worm retracts into the tube until the lip fits around the rim of the aperture like a collar (Fig. 5, C). In this position the lip acts as a mold and the sand grains which have accumulated onto the ventral lip surface are added to the end of the tube. At frequent intervals the ventral secretory surface of the worm is applied against the inner surface of the sand grains, laying down the inner secreted part of the tube and also securing the sand grain layer (Fig. 5, D).

Worms living in glass tubes commonly constructed partitions at various points within the tube. The partitions of *Ranzanides* do not have the distinct form and organization of those of other chaetopterids. The partition is merely a simple sheet of secreted material, often oriented obliquely across the tube (Fig. 5, F). Several perforations are present to permit the flow of water through the tube.

In laying down a partition the worm merely bends the anterior end ventrally across the tube and secretes a film of material in this position. There is no rotation of the anterior end to form a distinct disc as in other chaetopterids. The perforations are produced by the 4th setae. Following secretion of the partition, the worm flexes within the tube so that the 4th parapodia are at the level of the flexure. The body is twisted slightly to one side and the bundle of 5 heavy setae of the right fourth parapodium are thrust through the partition and then spread like a fan to widen the opening (Fig. 5, E).

The worm removes the partition by ripping it out. The fourth setae are used to tear away the partition at its junction with the tube wall. The body of the worm is then pushed through the opening. As in other chaetopterids, the 4th setae are also used to rip open the side of the tube wall in order to construct a new extent of tube. The old branch of the tube is then sealed off from the addition, thus always preserving what is essentially a non-branched straight tube.

Water is pumped through the tube of *Ranzanides* by the peristaltic action of the segments of the middle body region. Following the longitudinal contraction the diameter of a segment at the level of the parapodia is increased until the segment fills the tube (Fig. 5, I). Dorsally the two short finger-like notopodia fold over the mid-dorsal groove, protecting the groove and forming a dorsal margin of contact with the tube wall. The segment is then moved posteriorly like a piston driving water downward through the tube. The effective stroke occurs in an anteriorly directed wave with one segment moving downward after another. In the recovery stroke the diameter of the segment is greatly decreased with a resulting greatly lowered water resistance.

Pumping is more or less continual except when the worm is rapidly moving up or down the tube. But the strength and rate of pumping vary greatly and are dependent upon the rapidity of the peristaltic waves and the anterior-posterior length of the stroke. Although water is commonly driven through the tube to the posterior of the worm, reverse pumping was observed on a number of occasions, particularly when undesirable material had entered the tube.

Ranzanides is a very active worm. It can move rapidly up the tube, employing the anterior notopodia in a somewhat leg-like manner as is true of other chaetopterids. Also like other chaetopterids it frequently reverses position within the tube.

Ranzanides employs a mucous bag for feeding. A single bag is utilized and is secreted by the large wing-like second notopodia of the middle body region (Fig. 5, J). The bag is caught by the large cupule located halfway between the second and third parapodia.

The palps function as accessory feeding organs, conducting detritus particles along the major palpal groove to the mouth. But only small amounts of material were observed being obtained in this way. When a suspension of carmine particles was injected into a capillary tube containing a worm, the greater part of the suspension was ejected back out of the tube. A small amount was collected by the major groove of the palps and conducted downward to the mouth, and most of that which got past the palps was collected by the mucous bag.

Reverse peristalsis, or pumping, of the middle body region is apparently of primary importance in ridding the tube of any sudden invasion of undesirable material. In this way the greater part of an introduced carmine suspension was expelled. The ejection groove of the palps, however, still functions in removing large undesirable particles which enter the tube. The palps are also important in removing fecal waste. Fecal pellets released from the anus travel the dorsal ciliated groove along the entire length of the worm and then are expelled from the tube by the palps.

Mesochaetopterus taylori Potts

The author did not observe tube-building and feeding behavior in living specimens of *Mesochaetopterus,* which can be most easily collected along the west coast of North America, but preserved specimens of *Mesochaetopterus taylori* Potts from the coast of Washington were examined. When the structure of this species is compared to that of the other chaetopterids studied, a number of deductions regarding its tube-building and feeding behavior can be made.

Mesochaetopterus taylori inhabits a very long tube which is oriented vertically in the substratum. Although only fragments of tubes were examined by the author, the total length of the tube may exceed a meter and numerous collectors have attested to the difficulty of digging up intact specimens. The tube is composed of an outer layer of sand grains adhered to an inner secreted organic lining, but in large tubes the sand grain layer is often inconspicuous and the secreted layer has a parchment-like texture similar to the tube of *Chaetopterus.* Nothing can be stated regarding the presence or absence of partitions.

Species of *Mesochaetopterus* tend to be somewhat intermediate in size between *Chaetopterus* and the other chaetopterids. The single intact specimen of *M. taylori* examined by the author had a total length of 25 cm. The anterior body region (Fig. 1, E) is essentially like that of other chaetopterids. There are nine setigerous segments, with especially well-developed notopodia. The 4th notopodia carry a bundle of heavy setae like those of *Ranzanides.* The palps slightly exceed the anterior body region in length and bear both a ciliated gutter and an ejection groove.

The middle body region (Figs. 1, E and 5, K) displays marked resemblances to that of *Ranzanides.* The first pair of parapodia follow immediately behind the ninth and last notopodia of the anterior body region. It consists of a finger-like notopodium and an uncinate neuropodium. The second and third notopodia are aliform and each is followed by a large bivalved cupule. The dorsal surface of the body following the first three parapodia of the middle body region is strongly concave and transversely ridged. The remaining parapodia of the middle body region consist of short finger-like notopodia and uncinate neuropodia. Posteriorly the notopodia tend to become antenna-like and these segments may constitute a posterior body region as in *Ranzanides.* A dorsal groove runs the length of the body.

It is possible that *Mesochaetopterus taylori* constructs its sand grain-secreted tube in a similar way to that of *Ranzanides,* although the sand grain layer appears to be less important in *Mesochaetopterus.* The bundle of heavy 4th setae is at least employed to open the side of the tube wall. If partitions do exist, then these setae may be used to perforate them or remove them.

The water current passing through the tube is undoubtedly generated by peristaltic contractions of the 4th and remaining segments of the middle body region. These segments are virtually identical to the pumping segments of *Ranzanides.*

The presence of the two pairs of aliform notopodia, each followed by a large cupule, clearly indicates a feeding mechanism employing two mucous bags (Fig. 1, E). This conjecture has been confirmed by the observations of Dales at Friday Harbor. Potts (1914) claimed that when the animal is within its tube, the lateral margins of these three segments of the body are arched over toward each other, partially enclosing the dorsal surface. Within this enclosed space would lie the cupules (the over-arching lateral margins are not included in Figure 1, E in order that the mucous bags and cupules can be seen). The transversely ridged dorsal surface characteristic of these segments would line the enclosed tubular area, but the significance of the ridged surface is difficult to understand.

The secretion of two mucous bags is not characteristic of all species of *Mesochaetopterus. M. minutus* possesses but a single pair of aliform notopodia and one cupule, which indicates the formation of only one mucous bag.

Discussion

The members of the Chaetopteridae display a relatively uniform structure and behavior pattern with regard to tube-building and feeding mechanisms. The only atypical member is *Chaetopterus variopedatus,* which, being the most familiar species, has unfortunately tended to color the conception of the family for many zoologists.

The typical chaetopterid is a small worm inhabiting a more or less straight tube oriented vertically in the substratum. One opening of the tube projects above the surface of the sand or mud. A branching tube occurs in species of *Phyllochaetopterus* and in *Mesochaetopterus minutus,* but at least in *Phyllochaetopterus socialis* the branches, even when inhabited by another worm, tend to be sealed off from a main section. The tube is always composed of an organic secreted material. The secreted material is cornified in *Telepsavus* and *Spiochaetopterus.* In *Phyllochaetopterus, Mesochaetopterus,* and *Chaetopterus* it may be leathery or parchment-like. In species of two genera, *Ranzanides* and *Mesochaetopterus,* there is an outer layer of sand grains cemented to the secreted part of the tube. In all chaetopterids the tube is secreted by the convex ventral surface of the anterior body region.

A striking feature of the tubes of *Spiochaetopterus, Telepsavus, Phyllochaetopterus,* and *Ranzanides* is the presence of perforated transverse partitions. In *Spiochaetopterus* and *Telepsavus,* the partitions are always located near the bottom of the tube, and in an earlier paper (1964) the author suggested that the partition functions to prevent the collapse of the thinner-walled tube in this region. This may well be one function of the partition in these two genera; but it can not be the only function, for in *Phyllochaetopterus* and *Ranzanides* the lower part of the tube is not particularly delicate nor are the partitions always limited to this level.

Possibly the chief function of the tube partition is to modify the water pressure in some way within the tube. The condition of the tube in *Chaetopterus* would seem to support this conjecture. The tube of *Chaetopterus* lacks partitions, but the two openings of the tube have a much smaller diameter than does the greater part of the tube lying beneath the surface of the substratum. These differences in the tube diameter would reduce the speed of the water current and perhaps account for the absence of partitions in *Chaetopterus.* If partitions do function in modifying the pressure of the water current, then it is very likely that such partitions will be found to occur in the tubes of *Mesochaetopterus.*

The fourth notopodia of all chaetopterids carry heavy modified setae. These setae are used for cutting open the tube wall to permit the construction of a new branch or extent of tube, and they are also used for removing partitions and sometimes for perforating partitions. In *Spiochaetopterus, Telepsavus* and *Phyllochaetopterus,* the 4th notopodium carries a single heavy truncate blade-like seta, which is adapted for neatly cutting out the more button-like partitions constructed by species of these genera. In other chaetopterids, the 4th notopodium carries a bundle of heavy spear-like setae which pierce and tear rather than cut.

As in most sedentary tubicolous polychaetes, chaetopterids are totally dependent upon a current of water passing through the tube. The water current brings in oxygen and food and also removes excreted and gaseous waste. The current is generated in two ways. In *Spiochaetopterus, Telepsavus,* and *Phyllochaetopterus,* the current is produced by the beating of cilia (probably membranelles) lining the ring-like openings created by the foliaceous notopodia of the middle body region. In *Chaetopterus, Ranzanides,* and *Mesochaetopterus,* the water current is produced by peristaltic contractions of the piston-like segments of the middle body region. There are three such segments in *Chaetopterus* (Fig. 1, D) and they are modified somewhat differently from the many pumping segments of *Ranzanides* and *Mesochaetopterus.*

The primary method of feeding in all chaetopterids is the straining of water through a mucous bag, a unique mechanism found in few other animals. The secretion of the bag is the function of certain notopodia of the middle body region. The number of bags secreted simultaneously varies. A single bag is employed by *Telepsavus, Ranzanides, Chaetopterus* (Fig. 1, D), and *Mesochaetopterus minutus.* Two bags are spun by *Mesochaetopterus taylori* and eight or more by *Spiochaetopterus* and *Phyllochaetopterus.*

A characteristic feature of the family is a pair of palps that arise from the anterior dorsal surface just behind the mouth. With the exception of *Chaetopterus,* the palps are heavy and long. In *Chaetopterus* the palps are very short and small (Fig. 1, D). Each palp is provided with a deep longitudinal groove or gutter, lined with downward beating cilia. It is possible that the palp and its ciliated gutter represent the ancestral means of obtaining food in the Chaetopteridae. This is the method of obtaining food in the Spionidae, a tubicolous family closely related to the Chaetopteridae. The evolution of the mucous bag for feeding in the chaetopterids permitted the utilization of finer food particles than could be obtained by the palps. The palps will play a minor accessory role in feeding in most chaetopterids, and in *Phyllochaetopterus* are perhaps as important as the mucous bag.

The principal function of the chaetopterid palps is that of ejection of fecal pellets and unwanted debris which enters the tube with the incoming water current. Fecal pellets are carried anteriorly from the anus along a mid-dorsal ciliated groove located to the medial side of the ciliated gutter of the palp. The cilia of the ejection groove carry the pellet to the distal end of the palp, which at the time of ejection projects from the tube opening at the surface. Similarly, large undesirable particles or objects which enter the tube are caught on the palp surface, transferred to the ejection groove, and conveyed back to the exterior.

The ejecting function of the chaetopterid palps is correlated with the straight vertical tube which these worms inhabit. Although such a tube is open at both ends, the lower end is buried in sand and mud. The downward-moving water current can leave the tube by passing into the interstitial spaces of the surrounding substratum, but large masses of debris or foreign objects, even if they passed the worm without interfering with its feeding and pumping behavior, would eventually clog up the lower end of the tube. Feces would also contribute to the clogging of the tube and must therefore be removed from the opposite end of the tube. The primary function of the palps is therefore to maintain an unobstructed tube.

The situation in *Chaetopterus* is quite different. The tube is U-shaped and provided with both an inhalant and exhalant surface aperture. There is no need to move undesired objects back out of the inhalant opening. Unwanted material which enters the inhalant opening of the tube is pumped through the tube and out the exhalant opening. Significantly, the palps of *Chaetopterus* are greatly reduced in size. The major groove is still present but the ejection groove is absent. Fecal pellets are also flushed out by the exhalant water current, and the mid-dorsal ciliated groove, which in other chaetopterids extends the length of the body as a means of transporting fecal pellets, functions only to carry food balls and extends from the cupule to the mouth. The peculiarities of *Chaetopterus* are clearly correlated with the U-shaped structure of its tube.

The chaetopterids and the spionids probably evolved from some common, ancestral tubicolous polychaete, having palps as organs for obtaining detritus as food. The spionids retained this function of the palps. The chaetopterids, however, shifted to a feeding mechanism in which the water current passing through the tube was strained through a mucous bag; the palps were employed for maintaining an unobstructed tube.

The chaetopterids appear to have evolved along three main lines, each of which should probably constitute a single genus. One line embraces *Spiochaetopterus, Telepsavus,* and *Phyllochaetopterus*, in which the water current of the tube is generated by the ciliary rings of the foliaceous notopodia. The second line includes *Ranzanides* and *Mesochaetopterus,* which have the tubes covered by an outer layer of sand grains and pump water through the tube by means of the peristaltic contractions of a large number of segments of the middle body region. The third line is represented by *Chaetopterus*. Here the tube is U-shaped with two openings to the surface, the palps are greatly reduced, and a water current is produced by the peristaltic contractions of three specialized segments. *Chaetopterus* is probably more closely allied to the *Ranzanides-Mesochaetopterus* line than to the *Spiochaetopterus-Phyllochaetopterus-Telepsavus* group.

SUMMARY

1. Tube-building and feeding were investigated in members of the chaetopterid genera, *Telepsavus, Phyllochaetopterus, Ranzanides* and *Mesochaetopterus*.

2. With the exception of *Chaetopterus*, all members of the family construct a more or less straight tube oriented vertically in the substratum. The tube contains one or several transverse perforated partitions.

3. The tube is secreted by the ventral surface of the anterior body region. The enlarged fourth notopodial setae are used to remove partitions or to tear open the side of the tube wall in order to construct a new section of tube.

4. In *Telepsavus* and *Phyllochaetopterus* water is driven through the tube by the beating of ciliary membranelles. The membranelles line the ring-like openings formed by the foliaceous notopodia of the middle body region. In *Ranzanides, Mesochaetopterus,* and *Chaetopterus* water is pumped through the tube by the piston-like action of segments of the middle body region.

5. A mucous bag is utilized for feeding in all chaetopterids. The number of bags varies from one to many and they are always produced by the middle body region.

6. Except in *Chaetopterus,* a pair of long palps arise from the dorsal side of the head. Each palp carries a large and a small ciliated groove. The large groove, in which cilia beat proximally, functions as an accessory feeding device. The more important smaller groove, in which cilia beat distally, provides for the ejection of fecal pellets and any undesirable material which enters the tube with the incoming water current.

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FACTORS AFFECTING FIREFLY LARVAL LUMINESCENCE[1]

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The mechanism of control of the adult firefly flash, a burst of light lasting approximately 0.2 second, has been studied intensively (Buck, 1948; McElroy and Hastings, 1955; McElroy and Seliger, 1961; Buck and Case, 1961; Case and Buck, 1963; Buck, Case and Hanson, 1963; Smith, 1963). The larva does not produce a flash, but rather a uniform, structureless glow lasting for seconds (Dahlgren, 1917; Buck, 1948). Histologically its light organs represent a considerably simpler system because they contain no tracheal end cells, cells which are present in the adult light organ and implicated in flash control (Dahlgren, 1917; Snell, 1932; Alexander, 1943).

When an adult firefly is subjected to falling oxygen concentration it remains dark for a short period. Then a dull glow spreads over the organ, gains in intensity, and then slowly declines to extinction. If air is readmitted during this "hypoxic glow" a brilliant pseudoflash is produced, lasting 500 milliseconds or longer (Snell, 1932). The tracheal end cell valve theory of pseudoflash control, proposed by Snell and reaffirmed by Alexander (1943), implied that adult firefly luminescence was normally oxygen-limited and that the pseudoflash was independent of neural activity. Hastings and Buck (1956) also concluded that central nervous activity plays no part in the pseudoflash of the adult. Carlson (1961) examined the pseudoflash of the adult in more detail and implicated neural activity as well as hypoxia.

In lampyrid larvae Buck (1948) and Hastings and Buck (1956) observed that low ambient oxygen induces an hypoxic glow and that subsequently increased oxygen tension elicits a pseudoflash, which resembles that of the adult. The present study of the larval pseudoflash response was initiated to determine in what respect the adult and larval pseudoflash differ. It was hoped the differences in turn could aid in elucidating the disputed role of the adult tracheal end cell in flash control.

MATERIALS AND METHODS

Larvae of the genus *Photuris* were the subjects of this study. They were collected in the early autumn and stored either in petri dishes on moistened filter paper at 4° C. or in dirt-filled dishes at room temperature. The experimental animal was secured ventral side up on a narrow glass spatula provided with silver stimulating electrodes. The paired stimulating electrodes were usually positioned on each side of the ventral nerve cord in the sixth abdominal segment by insertion

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through the intersegmental membrane between the sixth and seventh abdominal segments. The spatula was placed in the basal segment of a glass Y-tube which was 30 mm. long and 1 cm. in diameter. Oxygen and nitrogen were led into opposite branches of the Y-tube through paired two-way stopcocks which permitted rapid shunting of the oxygen from the animal. Gas mixtures were prepared from commercial compressed nitrogen and oxygen metered through two-stage reduction valves and calibrated Fishcher-Porter flow meters. Composition was checked by gas analysis with a Scholander 0.5-cc. analyzer. Commercial compressed nitrogen, referred to hereafter as "nitrogen," was found to contain no more than 0.05% oxygen which was considered to be a negligible amount. A photomultiplier tube (RCA 931-A) and dissecting microscope were positioned above the animal and both were shielded from stray light by black cloth.

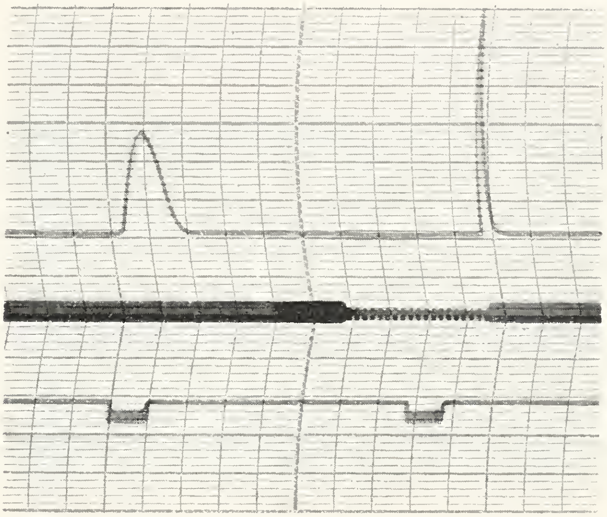

FIGURE 1. Stimulated glow and pseudoflash of *Photuris* larva. In this and all subsequent experiments except where noted: Upper trace is photomultiplier output; middle trace heavy line is 21% oxygen and narrow line is nitrogen; marks on narrow segment are time base, 1 mark per second, reading from left to right. Lower trace: stimulus, 5 volts, 20 msec. duration, 10 per second frequency. Electrode pair inserted in 6th abdominal segment in this and all subsequent experiments of larval light response.

In preparation for a pseudoflash one valve was rotated 180° to shunt off the oxygen and admit either nitrogen or a nitrogen-oxygen mixture. Rotation of this stopcock also opened a signal circuit. At an appropriate time the same valve was then rotated back 180° which allowed a higher concentration of oxygen to reach the animal suddenly and also closed the signal circuit. The pseudoflash was detected by a photomultiplier, the output of which was led to one or both channels of a Grass Polygraph. In some cases the amount of light recorded in a pseudoflash was obtained by integrating the light output with an integrating circuit utilizing a Philbrick operational amplifier.

RESULTS

1. *General characteristics of larval light responses*

The light induced in the larval organ by mechanically irritating the animal is variable in intensity and duration. An electrically stimulated response which mimics the mechanically induced light response is shown in Figure 1. A pseudoflash is also shown. The pseudoflashes of the larval and adult forms resemble each other more closely than do their respective natural light responses, which confirms the observation of Hastings and Buck (1956). The larval pseudoflash differs from the adult pseudoflash, which is illustrated in Figure 2 with a number of spontaneous flashes, in being more variable in duration and considerably longer, due to its increased decay period. Luminescence intensity is uniform over the larval lantern in all light responses.

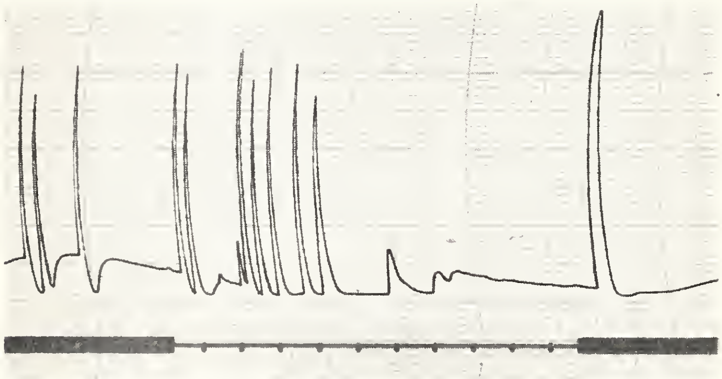

FIGURE 2. Spontaneous flashes and hypoxic glow followed by a pseudoflash in a *Photuris* adult male. Lower trace equivalent to middle trace in Figure 1. Hypoxic glow begins approximately 7 seconds after hypoxic onset; note its relatively low intensity. Note lengthening duration of adult spontaneous flash as anoxia proceeds.

2. *Effect of electrical stimulation and oxygen concentration on larval light responses*

Glow intensity in air is proportional to stimulation frequency up to approximately 10 stimuli per second, above which no further increase can be produced. Induced glow intensity also varies directly with oxygen concentration between 0% and 10% oxygen as shown in Figure 3. Whereas the adult will produce a glow in nitrogen, the larva cannot be induced to glow in this gas after the initial 15 seconds of perfusion. During continual stimulation glows can be maintained for long periods in oxygen concentration as low as 0.25% and the glow level responds to rapid alternation of oxygen concentration.

Larvae that have been glowing actively in air can produce pseudoflashes when the oxygen tension is manipulated. Non-glowing larvae must first be mechanically or electrically stimulated during the anoxic period before a pseudoflash can be produced by admitting oxygen. This stimulation during anoxia need not elicit glowing to be effective in pseudoflash production. Larvae left unstimulated for periods up to 20 minutes in nitrogen failed to produce a pseudoflash upon readmission of air, but would readily do so if stimulated during the anoxic period.

Like the glow in air, the maximum intensity of the larval pseudoflash is also a function of stimulus frequency. It reaches a maximum intensity at about 10 stimuli per second when the animal is stimulated during a period in nitrogen, as shown in Figure 4. The total light output of the pseudoflash was closely related to its maximum intensity. If stimulation is continued during readmission of air after hypoxia, an after-glow is produced on the falling phase of the pseudoflash, as shown in Figure 5. Pseudoflash intensity is proportional to oxygen concentrations at least up

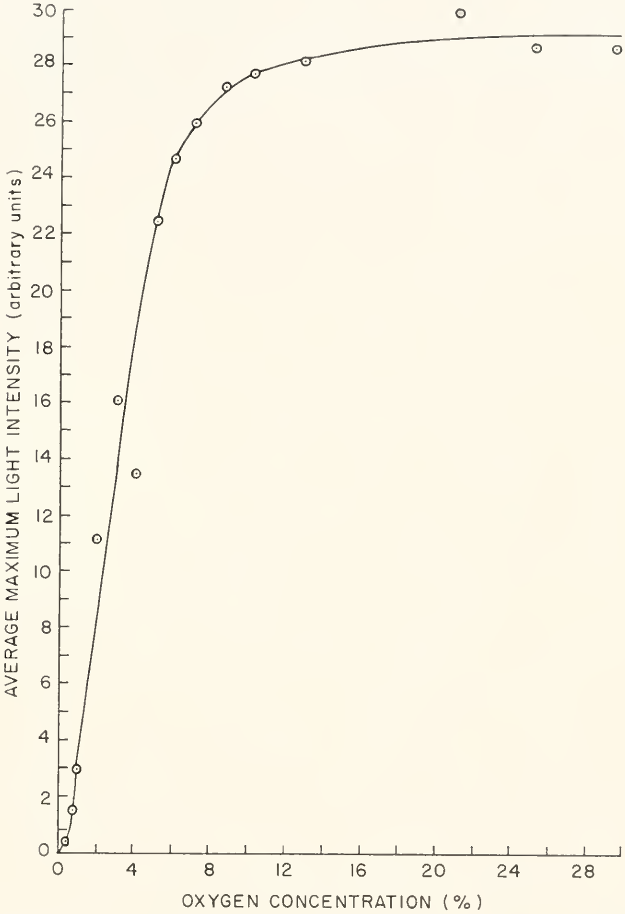

FIGURE 3. Effect of oxygen concentration on the maximum glow intensity attained during stimulation in one *Photuris* larva. Stimulus: 7 volts, 20 msec. duration, 10 per second frequency. Stimulation applied in various oxygen concentrations until a constant, maximum light intensity was attained. Recovery period in air was longer than 60 seconds. Oxygen concentration randomized during repeated experimental runs.

to 21% oxygen, as shown in Figure 6. The pseudoflash shape can be changed into a multi-peaked response with rapid alternation of 10% oxygen and nitrogen; see Figure 7.

3. *Effect of hypoxia duration on pseudoflash intensity*

With constant conditions of stimulation, pseudoflash intensity declines as the duration of hypoxia increases, as shown in Figure 8. Long hypoxic durations and

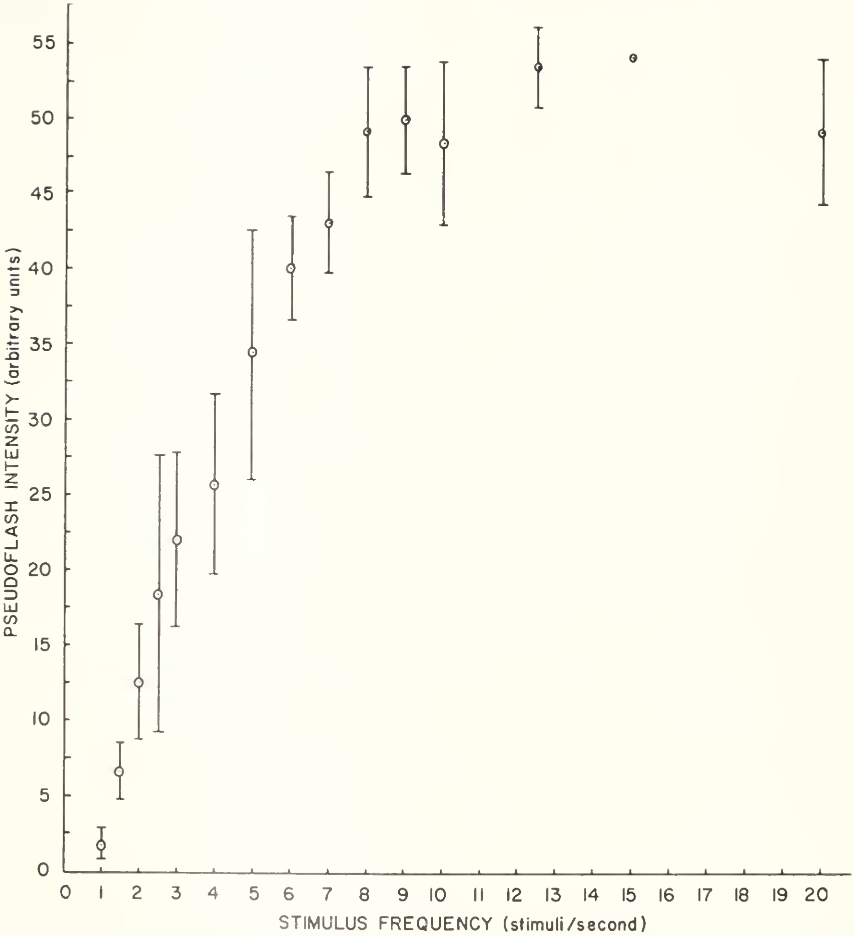

FIGURE 4. Effect of stimulus frequency during anoxia on pseudoflash intensity in *Photuris* larvae. Each point represents the mean pseudoflash intensity of 6 larvae except 2.5 stimuli per second which represents 4 larvae. Bars indicate ± 2 standard errors. All intensities are relative to the mean intensity obtained at 15 stimuli per second in the same individual in order to eliminate differences in geometry of light-collecting system. Stimulus frequency randomized during repeated experimental runs. Stimulus voltage constant for each larva, 20 msec. duration. Stimulation applied 5 seconds after hypoxic onset for a total duration of 5 seconds. Hypoxic duration 15 seconds. Recovery period between pseudoflashes lasted 45 seconds; nitrogen used during hypoxic period and pseudoflashes induced with 21% oxygen.

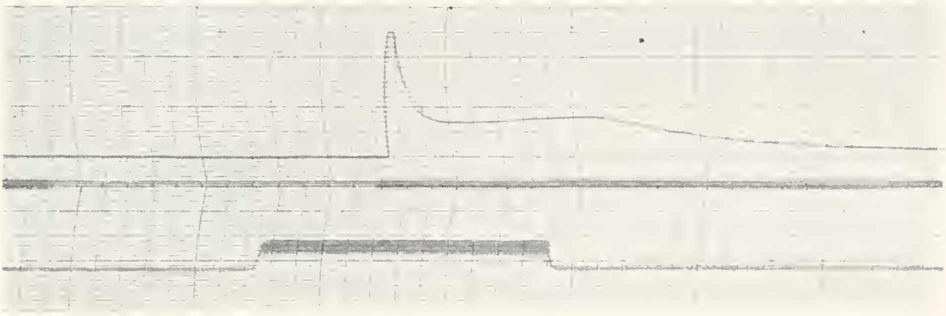

FIGURE 5. Effect of electrical stimulation prior to and during pseudoflash in *Photuris* larva. Lower traces stimulus, 4 volts, 40 msec. duration, 10 per second frequency.

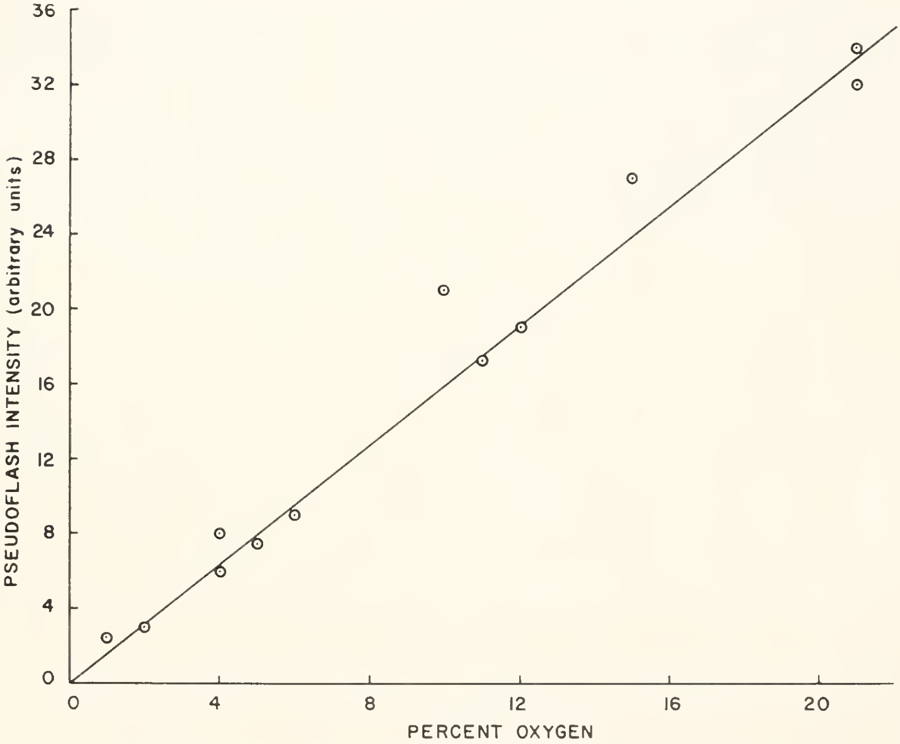

FIGURE 6. Effect of pseudoflash-inducing oxygen concentration on pseudoflash intensity in one *Photuris* larva. Stimulus 3 volts, 20 msec. duration, 10 per second frequency. Stimulation applied 5 seconds after hypoxia onset for a total duration of 5 seconds. Hypoxia duration varied from 16 to 23 seconds. Recovery period between pseudoflashes was 1 to 2 minutes. Nitrogen used during hypoxic period. Oxygen concentration randomized during repeated experimental runs.

their concomitantly reduced pseudoflashes did not affect the intensity of immediately following pseudoflashes induced after short hypoxia.

DISCUSSION

The similarities between the adult and larval pseudoflashes, with respect to induction sequence, response to electrical stimulation and response to oxygen concentration, suggest that both utilize the same basic process. In both developmental forms, the pseudoflash appears to be the result of an accumulation of light-producing substance and its rapid oxidation by the inrushing oxygen.

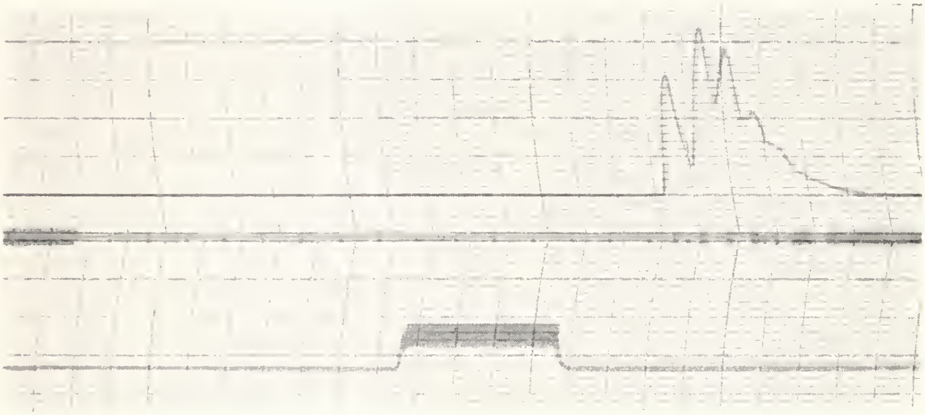

FIGURE 7. Effect of rapid alternation of 10% oxygen and nitrogen on the pseudoflash response of *Photuris* larva. Middle trace: heavy line, 10% oxygen; narrow line, nitrogen. Lower trace: stimulus, 4 volts, 40 msec. duration, 10 per second frequency.

There are a number of differences, however, between the responses of the two forms.

(1) Adults which are not flashing immediately prior to anoxia can produce a pseudoflash without stimulation during the anoxic period. Non-glowing larvae must first be stimulated in some fashion during anoxia before a pseudoflash can be elicited. This might suggest that it is hypoxia alone which triggers the hypoxic glow in the adult. However, spontaneous neural activity invariably occurs prior to onset of the hypoxic glow in the adult, as observed by Carlson (1962). The need to stimulate the larva then perhaps reflects a relative lack of spontaneous neural activity.

(2) The adult can maintain a glow in nitrogen for several minutes, indicating that oxygen is still available for the light reaction. The larva is apparently completely deoxygenated within 15 seconds in nitrogen because stimulation induces no glow after that period as anoxia continues. However, the anoxic larval photocytes are still responsive to electrical stimulation because stimulation initiated after the first 15 seconds of the anoxic period makes possible a pseudoflash upon readmission of air. This difference in time necessary to flush out the oxygen during exposure to anoxic gas may be a reflection of the relatively more complex tracheal supply of the adult (Buck, 1948). If this assumption is correct the observation

that the larval pseudoflash occupies, on the average, about six times the duration required for the adult pseudoflash cannot be explained on the basis of oxygen diffusion rates to the photogenic tissue. If diffusion rate controlled pseudoflash duration one would expect the adult pseudoflash to be of longer relative duration due to the evident impediment to oxygen diffusion noted in its resistance to deoxygenation. The explanation for the differences in pseudoflash duration between the two forms must lie, therefore, at another level in the luminescence process.

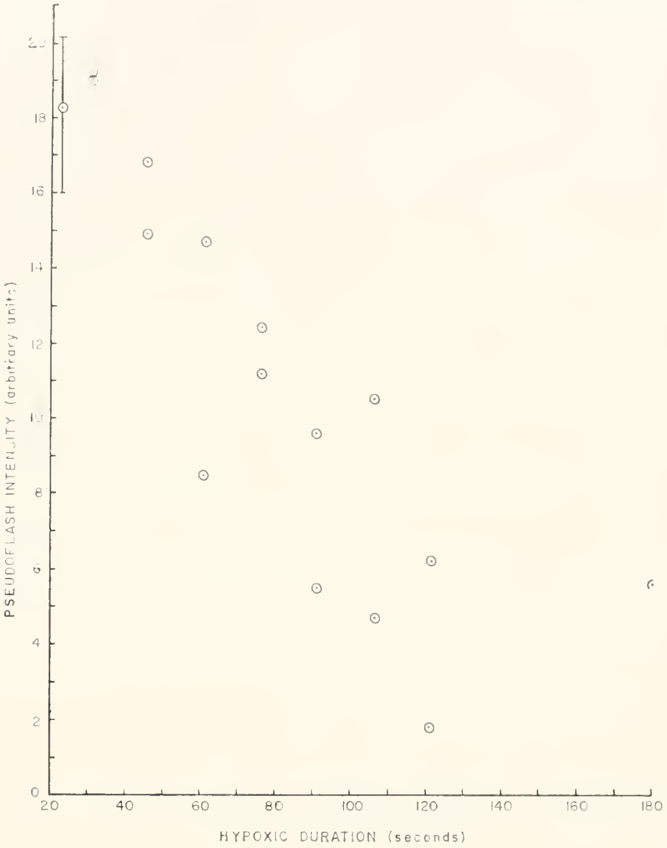

FIGURE 8. Decline of pseudoflash intensity with increasing hypoxia duration under constant conditions of stimulation in one *Photuris* larva. Stimulus: 6 volts, 4 msec. duration and 10 per second frequency. Stimulation applied 15 seconds after hypoxia onset for a total duration of 2 seconds. Recovery period between pseudoflashes was 60 seconds; nitrogen used to produce hypoxic period and pseudoflashes induced with 21% oxygen. First point is average of 34 measurements; line shows total range of values for that point.

(3) As illustrated in Figure 2, as anoxia proceeds the adult spontaneous flashes decline in intensity and increase in duration and then are replaced by a low level glow. Further, this shift from flash response to hypoxic glow fails to develop above an oxygen concentration of about 2.5%; instead the adult can continue to produce

small, spontaneous or electrically driven flashes (Hastings and Buck, 1956). The larval glow shows no such discontinuity but is simply proportional to oxygen concentrations below 10% under uniform stimulus conditions. One might explain these differences on the basis that the larval glow and the adult hypoxic glow are similar phenomena in that they represent processes which are oxygen-limited. Above about 2.0% oxygen, however, another limiting process may be superimposed upon the light reaction in the adult which results in a flash response. This non-oxygen-limiting process may involve the tracheal end cell or may be due to important biochemical differences within the photocytes of the larval and adult forms.

There is no comparable experimental evidence that the inactivation of light-producing substance by non-luminescent means, which may occur in the larva during anoxia, also occurs in the adult. Long anoxic durations, which apparently result in a large inactivation of substance by some dark reaction in the larva, do not prejudice the intensity of later pseudoflashes induced with shorter anoxic periods. It would appear that this non-luminescent inactivation of light-producing substance does not prevent reactivation for use in subsequent flashes.

SUMMARY

1. Electrically stimulated light responses and pseudoflashes were studied in larval fireflies, *Photuris* sp.

2. The larval pseudoflash is highly variable, but it is considerably longer in duration than the pseudoflash produced by the adult.

3. Larvae which were not previously glowing in air would not produce pseudoflashes unless stimulated during the anoxic period prior to admission of oxygen. Even with stimulation no glow could be produced in nitrogen. Pseudoflash intensity is proportional to stimulus frequency up to 10 stimuli per second. Light intensity is dependent on oxygen concentration up to 10% oxygen under uniform stimulus conditions. A multipeaked pseudoflash response can be obtained with rapid alternation of 10% oxygen and nitrogen.

4. Pseudoflash intensity declines with increasing hypoxic duration under uniform stimulus conditions.

5. Differences between the larval and adult pseudoflash response are discussed, but no difference could be linked directly to the operation of the adult tracheal end cell.

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THE MECHANISM OF THE SHADOW REFLEX IN CIRRIPEDIA. II. PHOTORECEPTOR CELL RESPONSE, SECOND-ORDER RESPONSES, AND MOTOR CELL OUTPUT[1]

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In a previous report (Gwilliam, 1963), certain electrical events at various locations in the nervous system of cirripedes, associated with changes in the light level impinging upon a photoreceptor, were described. At that time it was suggested that the photoreceptor cells have axons that do not synapse until reaching the supraesophageal ganglion, and that these cells influenced the activity of the second-order neurons by passive electrotonic conduction of a depolarizing potential which occurs when the photoreceptor is illuminated. Recent evidence from electron microscopy (Fahrenbach, 1965) supports this suggestion from a structural point of view, and observations to be reported here lend functional support.

Recent papers on the structure of the crustacean nauplius eye (Kauri, 1962; Elofsson, 1963) indicate that it is made up of three components, and the evidence presented here that adult balanid barnacles possess three (paired lateral and single median) photoreceptors suggests that they may well be derived from the three-parted naupliar eye found in the larvae of *Balanus* (Kauri, 1962). While it appears that the detailed structure of the three "compartments" of the larval medial eye does not coincide with that of the presumed separated components of the adult photoreceptors, the mere existence of three distinct components indicates a possible developmental source of the three adult photoreceptors.

In addition, further information has been obtained on neural pathways from the ocelli to certain of the muscles responsible for the withdrawal-closure response to a shadow that is so characteristic of most barnacles.

MATERIALS AND METHODS

Three species of barnacles have been used in this study. *Balanus eburneus* Gould was used for the intracellular recording, and the animals were supplied by the Supply Department of the Marine Biological Laboratory, Woods Hole, Mass. Other observations were made on specimens of *Balanus tintinnabulum* (L.) and *Balanus cariosus* (Pallas). The former were supplied by Dr. Eric Barham, Navy Electronics Laboratory, San Diego, California, and Dr. James Case, University of California, Santa Barbara. The latter were collected by the author from the north central Oregon coast.

The lateral eyes of *B. eburneus* were exposed by splitting the shell along the longitudinal (rostrocarinal) axis, carefully removing the opercular valves with the

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body of the animal attached, leaving two "half-shells," each of which bears a lateral eye and a short length of retinula cell axons. The eye is located at the junction of the fused rostrum-rostrolateral and lateral shell plates and is easily visible with the naked eye. These "half-shells" were then mounted, inner surface up, with soft wax, in the recording chamber. The pigmented mantle over the photoreceptor was then dissected away, and the capsule of the tapetum or reflecting layer was removed. This permitted direct viewing of the photoreceptor cells, which appeared as two orange-yellow areas in each eye, although histological examination reveals three cells.

Next, the preparation was treated with trypsin (× 300), 80 mg./100 ml. of sea water, for 45 minutes. Following this treatment, penetration with the micropipettes could be accomplished in many preparations. Attempts to penetrate cells without enzymatic pre-treatment were never successful.

Glass micropipettes filled with 3 *M* KCl, having a resistance of 10–15 megohms in sea water, served as electrodes. The amplifier used was an Argonaut "negative capacitance electrometer" which fed into a Tektronix type 502 dual beam oscilloscope.

Other recording techniques, the control of light to the preparation, and the making of permanent records are described elsewhere (Gwilliam, 1963). Light intensity is referred to as "unit intensity" or "intensity one" or a percentage of unit intensity achieved with neutral density filters. Unit intensity was approximately 1,000 foot-candles at the preparation.

The preparation used for external recording was achieved in the following manner: The opercular valves, bearing the body of the barnacle, were dissected free from the shell. This was then placed in a wax-lined dish, opercular plates down, and a pin thrust through the median junction of the apex of the scuta. The body was then extended along the longitudinal axis away from the terga and pinned. This exposed the mouth and ventral surface, brought the adductor muscle into view, and made dissection of the median photoreceptor, supraesophageal ganglion, and circumesophageal connectives relatively simple. In this position the lateral photoreceptors are found beneath the body of the animal close to the scutal margin just to either side of the mid-line in *B. tintinnabulum,* but would not be included in the preparation in *B. eburneus,* for in that species the eyes are displaced more basally and laterally onto the shell lining (see above).

The supraesophageal ganglion must be exposed by dissection, which then makes it possible to locate and identify the circumesophageal connectives, the antennular nerves (which contain the lateral ocellar axons) and the suprasplanchnic nerves. The area overlying the adductor muscle may be dissected away, which exposes the median ocellus and its nerve, the adductor muscle itself, and the great splanchnic nerve, with its adductor muscle motor branch. Further, one can expose the ventral ganglion and the cirral nerves to make the latter accessible for recording motor output. All this can be done without disrupting the circuit as illustrated in the diagram (Fig. 1), so that it is possible to record at any one site and remove sensory input as desired. Thus, one can record from cirral nerves or adductor muscle motor nerves with the rest of the system intact, cast a shadow, observe the effect, and then cut either the lateral or median ocellar nerve and again observe the effect of a shadow. The same procedure can be followed when recording from the circumesophageal connectives, but as it is necessary to cut them close to the ventral ganglion for recording, it would no longer be possible to record responses to shadows in cirral nerves or in the adductor motor supply.

RESULTS

Structure

After dissection as described above, the terga and the scutal apex would be at the bottom of Figure 1, with the cirri extending from the top. The general body surface viewed is morphologically the ventral surface. In most cases the median ocellar nerve can be seen through the thin, usually non-pigmented exoskeleton, and the "ophthalmic ganglion" of Darwin (the median photoreceptor) can sometimes be seen lying very close to the adductor muscle, at which point it is attached. It is also usually possible to see the great splanchnic nerve which originates on the dorsal aspect of the ventral ganglion, runs out laterally to the scuta, and gives off a motor branch that supplies the adductor muscle.

The diagram in Figure 1 is based on *B. tintinnabulum*, the same species illustrated by Darwin (1854, Pl. XXVII, Fig. 2), but apart from differences in orientation of the two figures, one significant difference should be noted. Darwin assumed (but did not actually see) a connection between the lateral ocelli and what he called the ophthalmic ganglion (the median photoreceptor of Figure 1) which I cannot find. It is clear that the lateral ocellar axons enter the supraesophageal ganglion independently of the median ocellar nerve, since severing the median ocellar nerve at the supraesophageal ganglion does not interfere with responses to shadows in the rest of the system as long as the antennular nerve is intact.

In a previous paper (Gwilliam, 1963), I stated that the median photoreceptor was probably the only one present in *B. cariosus*, and that it was only occasionally functional as a photoreceptor in *B. eburneus*. I am now convinced that both of these statements are in error, for lateral photoreceptors can be demonstrated physiologically in *B. cariosus*, if care is taken not to cut too close to the scuta when dissecting the opercular plates free. The small size of *B. eburneus* and consequent difficulty in dissecting make it likely that previously the median ocellar nerve was damaged in many preparations of that species.

These new observations, and the fact that *B. tintinnabulum*, *B. balanus*, *B. crenatus* and *B. balanoides* all possess both lateral and median ocelli convinces me that all balanids probably conform to the pattern illustrated diagrammatically in Figure 1, but that the lateral ocelli are better developed and more obvious in some than in others. *B. eburneus* and *B. amphitrite* are similar in having obvious, pigmented lateral ocelli in the position described for *B. eburneus* by Fales (1928). In *B. tintinnabulum*, *B. balanus* and *B. crenatus* they are not so obvious and lie closer to the mid-line, just inside the margin of the scuta in the opercular membrane. In *B. cariosus* and *B. balanoides* the lateral ocelli have not been seen, but can be physiologically demonstrated to occupy a position similar to that in the last three species mentioned.

The structure of the photoreceptors themselves is reported by Fahrenbach (1965) for the median ocellus of *B. cariosus* and, in less detail, for the lateral ocelli of *B. amphitrite* which are virtually identical to *B. eburneus*. Fales (1928) reports two large photoreceptor cells in each lateral eye of *B. eburneus*, but there are in fact three (based on examination of serial sections of *B. eburneus* lateral ocelli). In both the median and lateral ocelli examined, there is no ommatidial organization, and no evidence of a synaptic layer close to the ocellus. The cell bodies have finger-like "dendritic" projections which bear the microvilli, and each soma has a large axon that apparently does not synapse until the level of the supraesophageal ganglion. The size of the axons (15–20 μ in diameter) and the nature of the glial sheath around the ocellar nerve suggest a high value for the length constant of the axons.

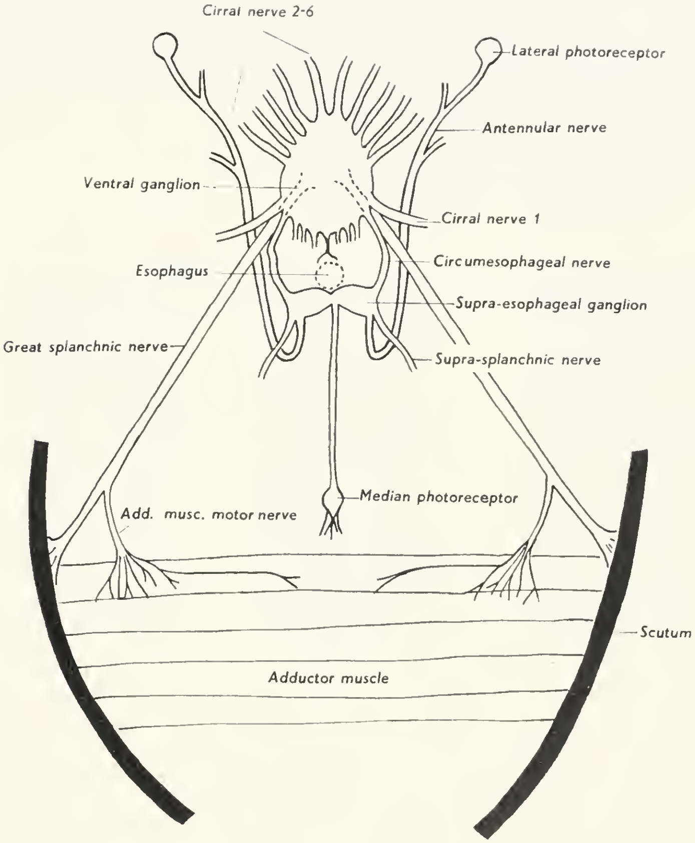
Cirral nerve 2-6
Lateral photoreceptor
Antennular nerve
Ventral ganglion
Cirral nerve 1
Esophagus
Circumesophageal nerve
Supra-esophageal ganglion
Great splanchnic nerve
Supra-splanchnic nerve
Median photoreceptor
Add. musc. motor nerve
Scutum
Adductor muscle

FIGURE 1. Diagram of the balanid central nervous system, showing the relationship of the photoreceptors to it. Based on *B. tintinnabulum*. Details of branching in the antennular nerve are schematic and are included simply to indicate that the nerve is mixed.

Electrical activity

(a) *Balanus eburneus:* intracellularly recorded responses

Although direct proof of penetration of photoreceptor cells is lacking, it was assumed when a maintained negative potential was recorded. Further, only those preparations which showed reversible depolarization when exposed to a light flash were assumed to have been successfully impaled. Such cells could often be held for as long as three hours, but relatively few such preparations were obtained. In the limited time available, only a total of twelve preparations met the above criteria for any significant length of time.

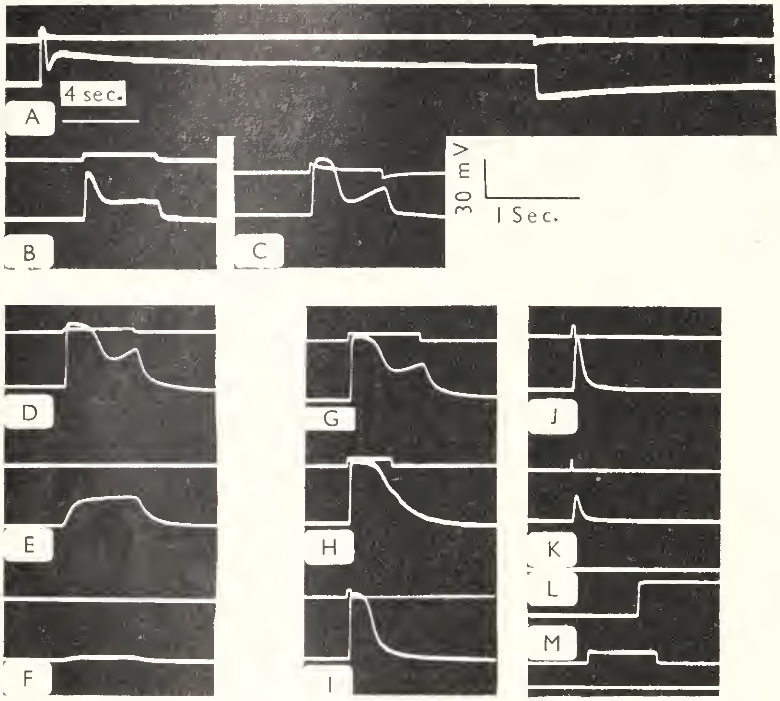

FIGURE 2. Intracellular records from the lateral photoreceptor cells of *Balanus eburneus*. Inset time calibration applies to B through M; voltage calibration applies to all records. In this figure and all others, upward deflection of the second beam indicates "on." A, sustained response. B and C, the response to a 0.8-sec. light flash, B at a lower intensity than C. D, response to a flash of unit intensity. E, 1.0% unit intensity. F, 0.1% unit intensity. Photocell failed to record in E and F. G–K, Decreasing time series. L, The membrane potential at the close of the time series. M, the effect of a light flash on the removed electrode.

In such cells, membrane potentials recorded varied considerably, depending on the immediate history of the penetrated cells. Initial membrane potentials recorded on penetration while viewing in relatively bright light were on the order of 30–45 mV, inside negative. After one hour in darkness these approached 60–70 mV.

The wave-forms of the potentials recorded when the preparation was exposed to a flash of light are shown in Figure 2. This consists of the familiar "on" transient, often, but not always, a secondary rise, followed by a maintained level of depolarization. At "off" this drops very close to the original membrane potential level (Fig. 2, A, B, C). Amplitude of the generator potential was graded in different light intensities (Figure 2, D, E, F), and the transient disappeared at low intensities. The wave-form also varied in light flashes of intensity one, but of different duration (Fig. 2, G–K). In this case only the transient remained in flashes less than 0.5 second in duration.

At the highest intensity the transient may overshoot zero potential, but this could not be determined with certainty, because of the shifting membrane potential dependent on previous exposure to light (*cf.* Naka, 1961; Naka and Eguchi, 1962a) and to the D. C. drift in the amplifier. However, in a dark-adapted preparation, the transient seldom exceeded 55 mV, which suggests that overshoot did not occur if membrane potentials reached the values of 60–70 mV which were recorded in other cells after dark adaptation.

It will be noted that these intracellular responses are very similar in form to the presumed intracellular response from the median eye of *B. cariosus* as previously reported (Gwilliam, 1963, p. 476) and very similar to the simple electroretinograms recorded from barnacle ocellar nerves, if the difference between A. C. and D. C. recording is taken into account. That is, the extracellularly recorded "mass" response is directly comparable to the single unit intracellularly recorded response, both being almost certainly uncomplicated by post-synaptic events.

Under the conditions of the observations reported here, it seems highly unlikely that the "on" transient has its source in other than the impaled retinula cell. The photoreceptor consists of three primary receptor cells, supporting cells, and very little else. There are no nearby post-synaptic cells to contribute, so the suggestion that the "on" transient originates elsewhere (Burkhardt and Autrum, 1960; Burkhardt, 1962) seems to be ruled out in this material. As Ruck (1964) points out, the recorded amplitude alone of the transient argues very strongly against its origin outside the retinula cell.

The records are also uncomplicated by anything resembling ordinary spikes. This is also true of the ocellar-nerve recorded ERG when the bundle is uncontaminated with other nerve fibers. There is thus no evidence that the retinula cell axons conduct ordinary spikes, despite the relatively great distances over which they presumably transmit.

It may be argued that in the illustrated cases the photoreceptor cell axons have been damaged in the exposure procedure, and that this could in turn destroy the spiking locus. However, if the "on" transient is accepted as an axonal event, its presence in these records argues against extensive axonal damage. It might also be argued that the light levels used are insufficient to operate the spiking mechanism, but the same light levels serve to inhibit firing of cells in the supraesophageal ganglion, proving that they are adequate to operate the normal post-synaptic inhibitory mechanism that leads to the shadow reflex upon release.

The suggestion put forth by Ruck (1964) that the transient may be a regenerative event has not been adequately tested in this material, but as Ruck himself points out, this will not account for sustained transmitter action on post-synaptic cells.

(b) External recording in *B. tintinnabulum* and *B. cariosus*

1. Lateral *vs.* median photoreceptor function

Having established that two morphologically distinct sets of photoreceptors existed, I tried to discover if they had different functions. The records reproduced in Figure 3 illustrate the results of observations on the two species. Figure 3, A illustrates a circumesophageal connective recording of the results of a shadow cast on a preparation of *B. cariosus* with both sets of photoreceptors intact. Figure 3, B was taken from the same preparation after severing the median ocellar nerve, and Figure 3, C after severing both antennular nerves. Figure 3, D is from a different preparation of the same species, the electrode recording from the motor supply to the adductor muscle, with both sets of photoreceptors intact. Figure 3, E is from the same preparation with the antennular nerves severed, and in Figure 3, F the median nerve has been severed as well.

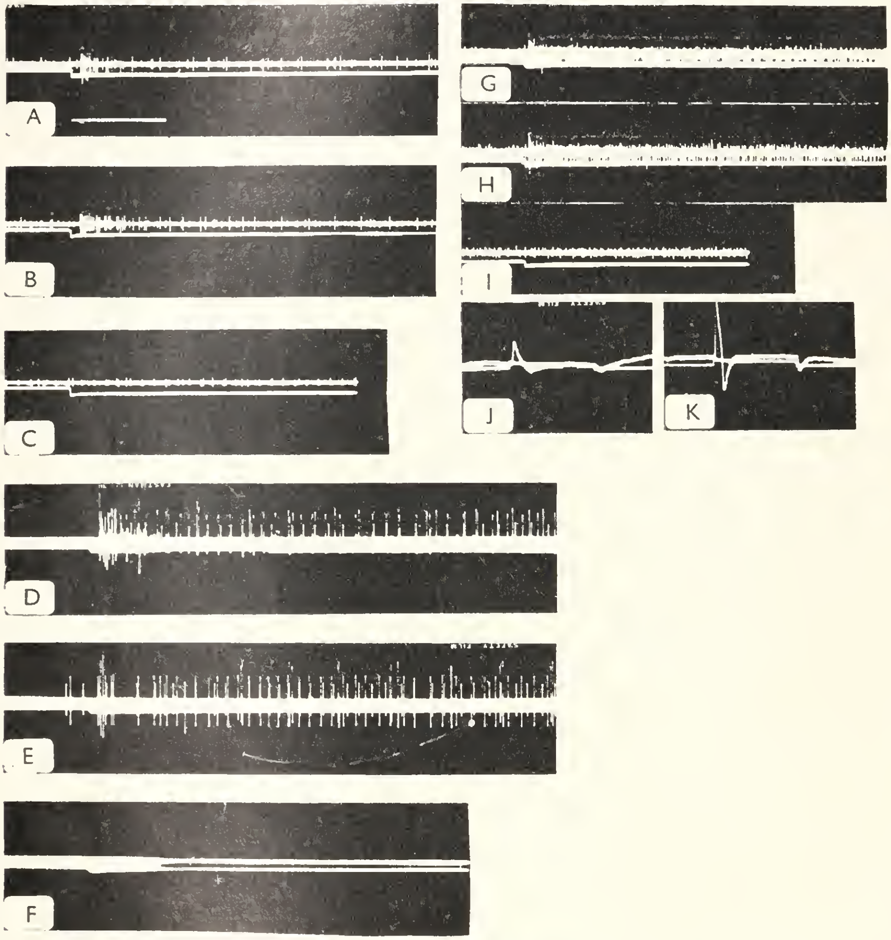

FIGURE 3. Function of the lateral and median eyes in *B. cariosus* (A–F) and *B. tintinnabulum* (G–K). See text for explanation.

These records prove the existence of lateral photoreceptors in *B. cariosus,* establish that both sets are capable of mediating the shadow reflex, and suggest that there is no difference in function between the lateral and median photoreceptors in this particular pathway.

Figure 3, G is a circumesophageal recording from *B. tintinnabulum* with both sets of eyes intact; in H, the antennular nerves have been cut; and in I, the median nerve was also severed. Recordings from the adductor motor supply give the same results as in *B. cariosus*. These records, also, indicate that there is no difference in function of the two sets of photoreceptors in *B. tintinnabulum*.

Figure 3, J and K were obtained from the cut ends of the nerves containing the photoreceptor axons. J is a record of the lateral ocellar ERG taken from the cut end of the nerve close to the supraesophageal ganglion, while K was recorded from the same relative position from the median nerve. Both records were taken at the same overall gain and band pass frequency (0.3–2000 cps) and at the same distance above the bathing medium. The difference in amplitude, rise and decay times in the two records may reflect the greater distance of transmission of the lateral ocellar axons (Fig. 3, J) and probably illustrates the decremental nature of ocellar axon transmission.

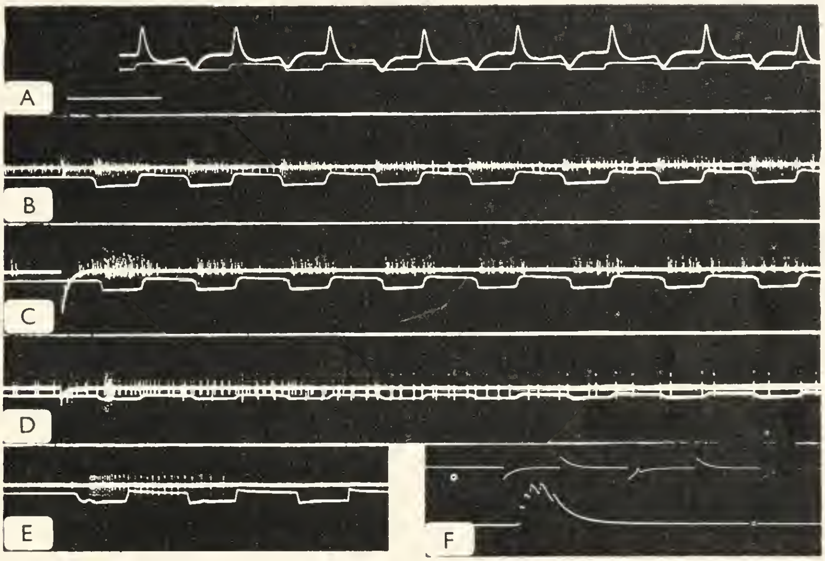

FIGURE 4. Series to illustrate different rates of adaptation to multiple stimuli at different points in the responding system. Upward deflection of upper beam in A and lower beam in F indicates *positivity* of active electrode. Membrane potential in F (indicated by the initial separation of the two beams) = 60 mV. In F, downward deflection of upper beam indicates "off."

It should be pointed out at this juncture that the initial deflection of the ERG when recording externally from the ocellar nerve at some distance from the photoreceptor with a single active electrode is positive in sign rather than negative as erroneously reported in Gwilliam (1963). If the record is taken just distal to, or from the region of the photoreceptor cells, the sign is reversed. This result then accords with other arthropod ocelli in which the ERG is cornea-negative and retinula cell axon-positive when recorded extracellularly, as shown by Ruck (1961) and others.

2. The response to multiple shadows

Gwilliam (1963) briefly reported that the response to multiple shadows at different points in the photoreceptor-motor output chain showed different rates of adaptation. This was investigated more fully in *B. tintinnabulum* and *B. cariosus*, two species obtained from rather different habitats and showing different behavioral reactions to multiple shadows. The similarities and differences between the two species are illustrated in Figure 4.

Figure 4, A illustrates the non-adapting nature of the ERG in *B. cariosus*, and identical records have been obtained from *B. tintinnabulum*. Figure 4, B is a record of a circumesophageal recording from *B. tintinnabulum* which illustrates that at this point (the presumed second-order neurons) adaptation is very slow, but will fail to follow after approximately 30 shadows of the duration and frequency shown. A very similar phenomenon can be demonstrated in the circumesophageal connective of *B. cariosus*. Similarly, the motor output to the cirri in *B. tintinnabulum* adapts very slowly (Fig. 4, C), but the cirral output in *B. cariosus* adapts very rapidly, often failing to follow even after a single shadow (Fig. 4, D), and seldom persisting for more than four shadows. In both species the motor output to the adductor muscle fails to follow after 1–4 shadows (Fig. 4, E of *B. cariosus*). Figure 4, F is a record of an intracellular response of one of the giant muscle fibers from the adductor muscle in *B. cariosus* and illustrates the effect of a burst of motor nerve action potentials on the muscle junctional potentials (*cf.* Fig. 4, E) in response to a shadow.

If one now turns to an intact, feeding animal and presents shadows of the same duration and frequency used in the neurophysiological work, the behavior of each species corresponds to the pattern seen in the records of Figure 4.

B. tintinnabulum will respond by withdrawal of the cirri and valve closure (adductor muscle contraction) to the first shadow. If shadow-casting is continued, the animal very quickly emerges, but continues to withdraw the cirri at each shadow, but after one or a few additional shadows fails to close the valves.

B. cariosus, on the other hand, responds to the first shadow, quickly re-emerges and, after one to four additional shadows, proceeds to execute "fishing" activities, completely ignoring the changing light level.

These responses, of course, will occur in this particular way only in the absence of any reinforcing stimuli such as mechanical shocks, or tactile stimuli. If the shadow is accompanied by a tactile stimulus or a blow to the dish containing the animals, they remain closed for much longer periods of time and do not adapt to the dual stimulus nearly so quickly.

While this difference in behavior is difficult to explain with any degree of confidence, it is interesting to note that the *B. cariosus* used in this study were collected from the outer Oregon coast where the wave action may be severe, and the water frequently contains much floating and suspended debris. *B. tintinnabulum*, however, was collected from harbor floats and pilings in relatively quiet bays in southern California. In the two differing situations, it may be that a shadow is a more "urgent" stimulus in quiet water (*i.e.*, more frequently signals the approach of a predator), and continued response is of significant value to the species. In more turbulent waters, where shadows quite often signal only a piece of floating debris, the response may be less significant.

Discussion

The information now available on the structure and function of the adult barnacle photoreceptors and the nervous system permits a résumé which represents a fairly complete description of, at least, the obvious pathways and events that are involved in the shadow reflex. In no case has the response chain, from photoreceptor cell membrane depolarization to muscle junctional potentials, been followed completely through in one species, but by combining information from several it is possible to reconstruct the probable chain of events.

It now seems highly probable that all balanid cirripedes possess two distinct sets of photoreceptors: a pair of bilaterally symmetrical lateral ocelli and a single median photoreceptor "ganglion." That these receptors contain retinula cells with typical arthropod rhabdomere microvilli is now established, and the absence of a synaptic layer close to the retinula cells is strongly indicated (Fahrenbach, 1965).

It is generally held that the adult cirripede eye(s) takes its origin from the median eye of the nauplius larva (*e.g.*, Doochin, 1951), and the structure of that eye in the larva of *Balanus* suggests the developmental source of the three separated photoreceptors found in the adult. While a detailed comparison of the structure of adult and larval eyes (Kauri, 1962; Fahrenbach, 1965) reveals considerable difference in numbers of sensory cells and their organization, the existence of three components in the larva is very suggestive. It should be recognized that many larval structures do not develop directly into adult structures but emerge as the definitive adult structures following a phase of larval "degeneration" (Bernard and Lane, 1962).

To judge from the location and structure of the two sets of "eyes" in an animal like *B. eburneus,* it would seem that the median photoreceptor receives light most easily when the animal has the opercular valves open and the cirri extended. However, it must be recognized that an actively "fishing" barnacle probably casts shadows on its own median photoreceptor, which suggests the existence of inhibitory feed-back mechanism to prevent withdrawal reactions during this process.

In the lateral photoreceptors of *B. eburneus*, the location of light-absorbing and reflecting pigments over the inner surface of the PR cells makes it apparent that they must receive light either parallel to the shell plates and/or from the outside. In an animal like *B. tintinnabulum* this may also be true, but the inner shielding is less well developed. Very little can be said about *B. cariosus* lateral eyes, for the structure has not yet been morphologically identified.

The structural information on the retinula cells provided by Fahrenbach (*loc. cit.*) helps a great deal in explaining the absence of propagated action potentials in barnacle retinula cell axons. The large size of these axons (15–20 μ diameter) contrasts markedly with those of, for example, the cockroach dorsal ocellus which averages 0.5 μ (Ruck, 1964). Also, the inter-axonal space which is filled with glial cell membrane is quite large, so that in contrast to the cockroach, the length constant of barnacle retinula cell axons is probably quite large. It thus appears that a structural basis for long-distance electrotonic conduction is present.

The function of these photoreceptors seems to be primarily that of initiating the shadow reflex, although other functions may also be imagined. Structural considerations rule out any image-forming capabilities, and it seems evident that the "eyes" are relatively simple light-level and transient-photic-event monitors. No difference in function of the lateral and median photoreceptors is so far apparent with the techniques used in this study, but more subtle functional differences are not precluded.

In a purely speculative manner, one might imagine the sequence of events leading to a shadow reflex as occurring in the following way: self-cast shadows on the median photoreceptor would cause a certain amount of depolarization in the second order neurons at "off." In an immobilized preparation this would be sufficient to trigger the reflex, but in a "fishing" animal this would be countered by inhibitory neurons (acting on the same second-order cells) activated by the body movements. The balance between these two processes and the inhibitory influence of the illuminated lateral photoreceptors would serve to keep the second-order cell membrane potential depressed below the firing level. If, during this process, the added depolarization (release from inhibition) furnished at "off" by the lateral photoreceptors should impinge on the second-order neurons, the firing level would be reached, which would operate the withdrawal-closure reflex; this would in turn shut off the inhibitory feed-back mechanism and keep the animal contracted until the shadow was removed, or until firing in the second-order cells ceased (a matter of approximately 30–60 seconds).

That there are distortion-sensitive sensory cells present in the mantle lining close to the body can be demonstrated by stretching the mantle while recording from the cut end of the antennular nerve, the same nerve that carries the lateral photoreceptor axons. The spikes shown in Figure 10 (page 482) of the previous paper (Gwilliam, 1963) are almost certainly in this category. Whether or not they inhibit the same second-order cells or the motor cells involved in the shadow reflex is not known. Hoyle and Smythe (1963) have been unable to demonstrate peripheral inhibition in barnacles, but central inhibition certainly occurs.

However, the chain of events as demonstrated to date suggests the following interpretation:

(a) The photoreceptor cells generate a sustained depolarizing potential when illuminated.

(b) This potential is transmitted by passive electrotonic conduction *via* the large retinula cell axons to the supraesophageal ganglion where the axons synapse with the second-order neurons.

(c) The sustained depolarization probably causes the continual release of an inhibitory transmitter substance from the terminations of the retinula cell axons which prevents the second-order cells from firing, although inhibition may be accomplished by some other mechanism.

(d) At "off," the inhibition is released and the second-order cells begin to fire.

(e) The second-order cells synapse either directly or through other interneurons in the ventral ganglion with motor neurons. At this level the post-synaptic event is excitatory.

(f) At this level, rather than the previous one, the phenomenon of synaptic failure (seen as a failure of motor neurons to respond to multiple stimuli) probably occurs. This "tendency to failure" varies in different species and in different motor cells of the same species.

It thus appears that the barnacle retinula cell behaves in a very similar fashion to the retinula cell in the insect dorsal ocellus (Ruck, 1962, for summary), the striking difference being the greater distance between the retinula cell and the first synapse. This has apparently been compensated for in the barnacle by the morphological specializations referred to previously rather than by the development of an impulse-propagating mechanism. It seems quite plausible to argue that this mode of transmission is fundamental to arthropod photoreceptor cells, and the existence of a spiking mechanism (Naka and Eguchi, 1962b) represents a high degree of specialization. Washizu (1964), recording intracellular potentials from blowfly compound eyes, detected no impulse activity and demonstrated that the "on" transient did not overshoot zero potential and was graded. Unequivocal evidence of propagated impulse activity in retinula cell axons, on the other hand, is very limited.

SUMMARY

1. The gross structure of the balanid central nervous system and some of the peripheral structures involved in the shadow reflex are described and figured (Fig. 1). The existence of both paired lateral and single median photoreceptors in several species of barnacles is established, and is probably true for all balanid cirripedes.

2. Intracellular sensory potentials from the lateral ocelli of *B. eburneus* indicate that spiking does not occur in these retinula cells, and that the wave form of the response to a light flash is very similar to comparable records from other arthropod retinula cells.

3. No significant difference between the function of the lateral and the median ocelli has been shown with the procedures used in this study.

4. The different rates of adaptation of neurons in the reacting chain have been studied. The primary sensory event is non-adapting, the presumed second-order neurons adapt very slowly, as does the cirral motor output in *B. tintinnabulum*. The cirral output in *B. cariosus*, however, adapts rapidly, and so does the adductor muscle motor output in both species. This difference in motor output correlates very well with the behavior of intact animals.

5. The probable chain of events leading to the withdrawal-closure response to a shadow is summarized.

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OBSERVATIONS ON THE NUTRITION OF MONOGENETIC TREMATODES

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Relatively little information is available regarding the general pattern of nutrition in the Trematoda Monogenea, but there are indications that the two sub-orders of this class of parasitic flatworms differ considerably as regards the nature of their diet. The Monopisthocotylea so far investigated are reported to feed on the epidermal tissues and associated secretions of the host organism, whilst the Polyopisthocotylea appear to be largely sanguinivorous and take in little host tissue other than blood (Goto, 1895; Heath, 1902; Folda, 1928; Gallien, 1934; Sproston, 1945; Llewellyn, 1954; Jennings, 1956, 1959; Uspenskaya, 1962; Kearn, 1963).

Other differences between the two sub-orders, concerned with nutrition, are seen in the cellular structure of the digestive organs. Thus, in the Monopisthocotylea the intestine is lined by a continuous and unpigmented gastrodermis; but in the Polyopisthocotylea the gastrodermis is typically discontinuous and consists of columnar cells, containing varying amounts of brownish or black pigment, interspersed with areas devoid of cells and consisting only of thin basement membrane (Baer and Euzet, 1961). In a number of species the pigment has been identified as hematin, a degradation product of hemoglobin (Llewellyn, 1954; Jennings, 1959).

These differences in gastrodermal structure within the Monogenea are presumably related to the differences in diet and they may reflect, also, further differences in the site and course of the digestive process. In the present investigation, therefore, the relationships between diet, gut structure and digestion in the Monogenea have been studied, as part of a comparative survey of nutrition within this class of Trematoda.

MATERIALS AND METHODS

The following species of Monogenea, listed systematically with details of their hosts and parasitic locations, have been examined:

MONOPISTHOCOTYLEA

Calicotyle kröyeri Diesing. Cloaca of the thorn-back skate, *Raia clavata* and the starry ray, *Raia radiata*.

Entobdella hippoglossi Müller. Skin and general body surface of the halibut, *Hippoglossus hippoglossus*.

Udonella caligorum Johnston. Egg sacs of copepods (*Caligus* sp.) found on the head and in the buccal cavity of the cod, *Gadus callarias*.

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POLYOPISTHOCOTYLEA

Polystoma integerrimum Fröhlich. Urinary bladder of the common frog, *Rana temporaria.*

Diplozoon paradoxus Nordmann. Gills of the minnow, *Phoxinus phoxinus.*

Discocotyle sagittata Leuckart. Gills of the trout, *Salmo trutta.*

Diclidophora merlangi Kühn. Gills of the whiting, *Gadus merlangus.*

Octodactylus palmata Leuckart. Gills of the ling, *Molva molva.*

Plectanocotyle gurnardi Beneden & Hesse. Gills of the grey gurnard, *Trigla gurnardus.*

To determine the food of each species, and to study the structure of the gut, specimens were fixed in Bouin, Susa, or 10% formalin immediately after removal from the host, and serial sections cut at 5 μ after impregnation and embedding in polyester wax (m.pt. 37° C.) or paraffin wax (m.pt. 56° C.). For identification of intestinal contents sections were examined by one or other of the following methods:

1. The alcian blue method for mucins (Steedman, 1950).
2. The periodic acid-Schiff (P.A.S.) method for mucins and carbohydrates.
3. The mercuric bromphenol blue method for proteins (Mazia, Brewer and Alfert, 1953).
4. The benzidine method for hemoglobin (Pickworth, 1934).
5. The application of various solubility and bleaching tests for hematin (summarized by Jennings, 1959).
6. The Gmelin test for hematoidin and bile pigments.
7. The Turnbull's and Prussian blue methods for ferrous and ferric salts.
8. Various routine histological methods, *e.g.,* hematoxylin and eosin, Mallory's trichrome stain, Feulgen's reaction for nuclei, etc.

To aid identification of the chosen food the host organs were fixed and examined by the above methods, for comparison of tissue components with the trematode's intestinal contents. Further, where the trematodes had obviously only recently fed, they were induced to regurgitate the food, by gentle pressure, and the material so obtained examined either fresh or after treatment as a fixed and stained smear.

In the study of the feeding mechanisms the trematodes were observed alive upon their hosts, whenever this was possible, and others were fixed and sectioned *in situ.* The latter process was facilitated by fixation in warm (40° C.) Bouin, or by plunging the host organ and attached flatworms into isopentane, cooled to − 160° C. in liquid nitrogen, followed by transfer of the frozen mass into fixative held at − 1° C.

The site and course of digestion were investigated by isolating recently fed trematodes in aerated salt or fresh water (Hédon-Fleig saline with added glucose for *Polystoma*) and fixing individuals at progressive intervals up to three days, the maximum survival time for most species. The progressive breakdown and absorption of the food was followed in sections prepared and treated as above. Enzyme activity in the alimentary system was investigated histochemically, using frozen or 45° C. paraffin wax sections prepared after fixation at − 1° C. in 10% formalin buffered to pH 7.0. The histochemical methods employed included the indoxyl acetate method for non-specific esterases (Holt, 1958), both metal-salt and azo-dye methods for alkaline and acid phosphatases (Gomori, 1952; Burstone, 1958), the

Tween 80 method for lipase (Gomori, 1952) and the L-leucyl-β-naphthylamide method for leucine aminopeptidase (Burstone and Folk, 1956).

Observations

MONOPISTHOCOTYLEA

1. *Calicotyle kröyeri*

Calicotyle kröyeri feeds exclusively on epidermal cells and mucoid secretions derived from the lining of the skate cloaca. In many instances the gut lumen of specimens fixed immediately after removal from the host contained mucus, staining strongly with alcian blue and P.A.S., together with numerous large cells 10–12 μ in diameter and containing prominent nuclei (Fig. 1). These cells are identical with the epidermal cells *in situ* on the cloacal wall or lying free in the mucoid material coating the walls of the cloacal chamber.

The mouth in *C. kröyeri* is anterior and ventral, and surrounded by a poorly defined oral sucker. The anterior lip of the sucker contains unicellular glands whose secretions are used for adhesion to the cloacal wall, and the posterior portion bears a tongue-like valve which on contraction cuts off the cavity of the sucker from the rest of the alimentary system (Fig. 2). The pharynx is highly muscular and devoid of gland cells, and is used to suck in the semifluid mucus and desquamated epidermal cells which are always present in the cloaca. The cloacal wall and its epidermis are always intact and undamaged, even when many specimens of *Calicotyle* are present, and it appears that the pharynx never removes living epidermal cells or breaches the epidermis.

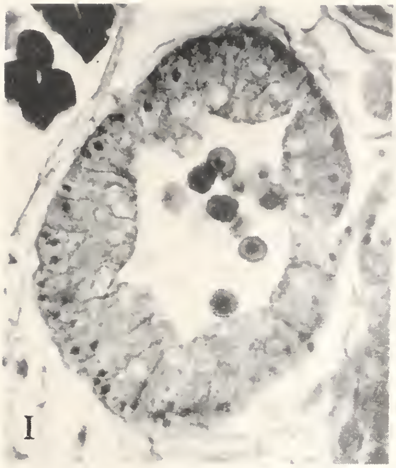
The pharynx leads *via* a short esophagus into the intestine, which consists of two simple unbranched ceca. The esophagus is surrounded by many acidophilic gland cells (Fig. 2) which open into its lumen, but the function of their secretion remains unknown.

The intestinal ceca are lined by a single-layered continuous gastrodermis, made up of columnar cells 16–18 μ tall and 6–8 μ wide, with granular cytoplasm and basal vesicular nuclei (Fig. 1). The cells go through a secretory cycle in which a small vacuole appears basally and then increases in size as it moves to the distal portion of the cytoplasm. The vacuoles eventually pass out into the gut lumen where they may remain as visible and discrete structures for varying periods before they finally disappear.

The entire gastrodermis consistently shows a strongly positive reaction for non-specific esterases, apart from the vacuoles whose finely granular contents remain unstained (Fig. 3).

The mucoid and cellular elements of the food are progressively homogenized as they lie in the gut lumen, demonstrating the occurrence of extracellular digestion. The enzymes responsible for this originate, presumably, in the esophageal glands and from the vacuoles released by the gastrodermis. No inclusions were seen in the gastrodermal cells, apart from the vacuoles, but the intense esterase activity seen in the cytoplasm suggests the occurrence of some intracellular digestion following absorption of partially digested material from the gut lumen.

Non-specific esterases are found also in the cuticle, notably that of the anterior ventral region and that lining the oral sucker, and may be used by the trematode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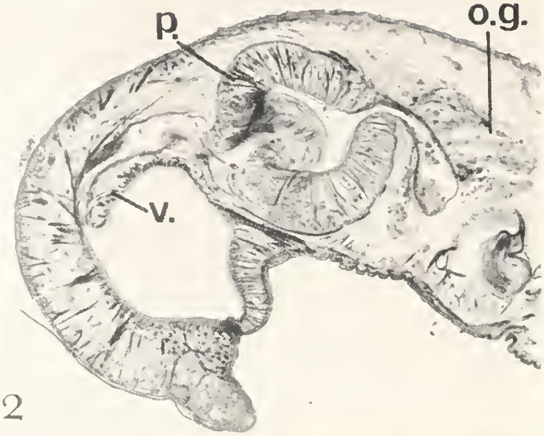

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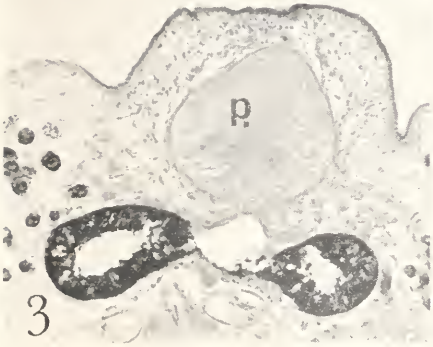

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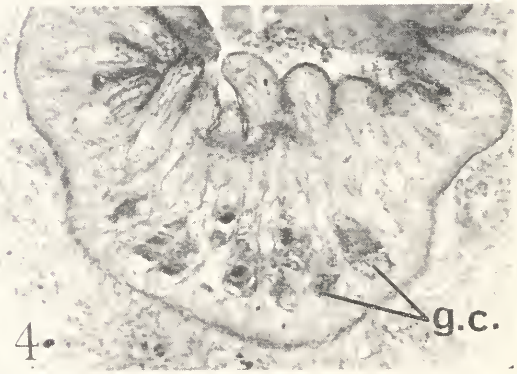

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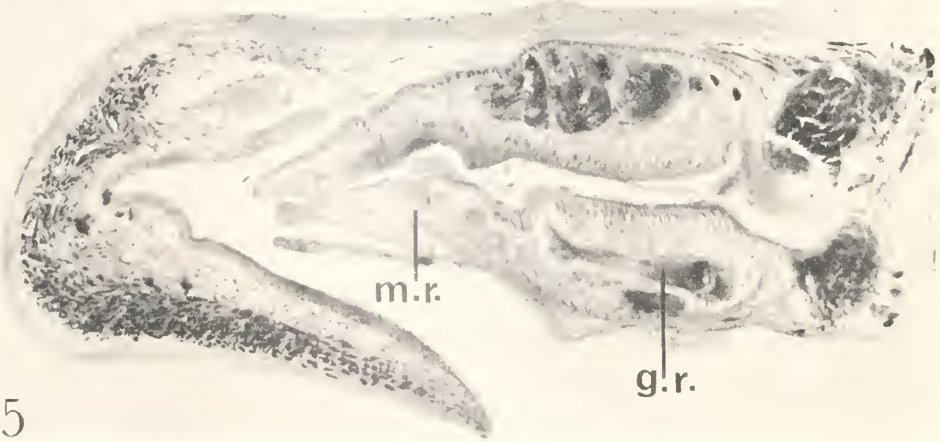

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FIGURE 1. *Calicotyle kröyeri.* Transverse section of an intestinal cecum showing the vacuolated continuous gastrodermis and, in the lumen, recently ingested host epidermal cells. Hematoxylin, eosin and alcian blue. Scale: 1 cm. = 20 μ.

in some form of extracorporeal digestion to accelerate the sloughing-off of spent cells from the cloacal epidermis.

2. *Entobdella hippoglossi*

Examination of the intestinal contents of *E. hippoglossi* fixed immediately after removal from the host showed that in this species, as in *Calicotyle,* the food consists entirely of host epidermis and mucus, and no traces were found of ingested dermal tissue components such as chromatophores or blood cells.

The ventral subterminal mouth leads directly into the pharynx, which is considerably modified from the usual trematode type to form a muscular-glandular feeding organ (Fig. 5). The anterior portion of this organ is entirely muscular, with flexible lips, and can be protruded through the mouth for application to the host epidermis. The posterior portion is glandular and consists of 40–50 large acidophilic gland cells, separated from each other by muscle fibers. Each cell communicates individually with the lumen of the anterior part of the feeding organ by a fine duct, and each duct opens distally at the apex of a large papilla (Fig. 4).

The gland cells give no reaction with the indoxyl acetate method for non-specific esterases, but fresh frozen sections applied to thin films of solidified 2% aqueous gelatine cause liquefaction and cavitation in the area covered by the glandular portion of the feeding organ, indicating the presence of a proteolytic enzyme. The feeding organ of *E. soleae* is reported to produce a similar gelatine-splitting protease (Kearn, 1963), and it seems likely, therefore, that protease production in the pharynx is characteristic of the entobdellid trematodes as a group.

It was not possible to observe *E. hippoglossi* in the act of feeding, but the presence on the host skin of circular lesions of the approximate diameter of the feeding organ indicates that the proteolytic secretions are used to erode and dissolve epidermal tissue prior to ingestion. This is supported by the fact that relatively few intact epidermal cells are found in the intestinal contents, even when the gut is full and the trematode obviously only recently fed. Generally the gut contents are quite homogeneous, acidophilic and stain only lightly with alcian blue or P.A.S., in marked contrast to the situation seen in *Calicotyle.*

The feeding organ leads posteriorly into the intestine *via* a short esophagus, into which open the ducts of numerous unicellular glands lying in the parenchyma of the anterior portion of the body. These esophageal gland cells are intensely basophilic but the function of their secretion could not be detected.

FIGURE 2. *Calicotyle kröyeri.* Longitudinal section through the anterior region. o. g., oesophageal glands; p., pharynx; v, tongue-like valve which can close off the pharynx from the oral sucker. Mallory. Scale 1 cm. = 200 μ.

FIGURE 3. *Calicotyle kröyeri.* Horizontal longitudinal section of the anterior region, showing the pharynx (p.) and portions of the two intestinal ceca. The gastrodermis in each cecum shows intense non-specific esterase activity. Holt indoxyl acetate method. Scale: 1 cm. = 50 μ.

FIGURE 4. *Entobdella hippoglossi.* Transverse section through the posterior glandular region of the feeding organ, showing the gland cells (g. c.) and papillae. Mallory. Scale: 1 cm. = 75 μ.

FIGURE 5. *Entobdella hippoglossi.* Longitudinal section through the anterior region showing the muscular-glandular feeding organ. g. r., glandular region; m. r., muscular region. Hematoxylin and eosin. Scale: 1 cm. = 125 μ.

The intestine is divided into two ceca which re-unite posteriorly by means of a commissure and give off over their entire length branched diverticula. It is lined throughout by a continuous gastrodermis consisting of uniform flattened cells, 12–15 μ long and 5–7 μ tall, with finely granular cytoplasm and basal vesicular nuclei. Gland cells are absent and no enzyme activity could be demonstrated. The only variation observable in the gastrodermis is in the height of the constituent cells, and this is related to the amount of food present in the lumen, the cells becoming even more flattened as the intestinal walls stretch to accommodate newly ingested material.

The amount of material in the gut lumen decreases with time, after feeding, but without noticeable change in consistency from the relatively homogeneous condition in which the food is ingested. This fact, together with the absence of gland cells from the gastrodermis, suggests that the bulk of digestion in *E. hippoglossi* is effected by the secretions poured on to the food from the glands of the feeding organ before and during ingestion, aided perhaps by the secretions of the esophageal glands. The gastrodermis would thus appear to be entirely absorptive in function and to play little or no part in the production of the digestive juices.

3. *Udonella caligorum*

Udonella caligorum lives attached to the egg sacs of copepods (*Caligus* sp.) which in turn are ectoparasitic in the buccal cavity and on the head region of cod, halibut and ling.

The only recognizable material found amongst the gut contents of *Udonella* was a mucoid substance staining lightly with alcian blue and P.A.S., and often the intestine contained only a finely granular acidophilic digest. Nothing can be seen to suggest that *Udonella* feeds on the copepod tissues or body fluids, and it is concluded that the trematode ingests mucus, and perhaps sloughed-off epidermal cells, from the fish skin or mucous membrane adjacent to the copepod's point of attachment.

The mouth in *Udonella* is anterior and ventral, and leads directly into the large muscular pharynx. This can be protruded slightly through the mouth but is not armed or equipped with glandular elements so that it is unlikely that it penetrates host tissues. Feeding, therefore, is probably a case of merely sucking in the material lying on the fish epidermis.

The intestine in *Udonella,* in contrast to that in most other Monopisthocotylea, is undivided and extends almost to the posterior end of the body as a simple sac, reminiscent of the sac-like gut of many rhabdocoel Turbellaria. It is lined by a flattened and continuous gastrodermis similar to that found in *Entobdella.*

Digestion appears to be entirely intraluminar, judging from the appearance of the gut contents, and nothing was seen to indicate intracellular digestion, as the gastrodermis shows no trace of esterase activity.

POLYOPISTHOCOTYLEA

1. *Polystoma integerrimum*

Polystoma integerrimum is sanguinivorous, feeding on blood drawn from the capillaries of the frog urinary bladder, and no host tissues other than blood were found in the gut contents.

The ventral mouth is encircled by an oral sphincter and leads into the cavity of the oral sucker. This is lined by cuticle and surrounded by numerous unicellular glands which open *via* long branched ducts over the external surface of the sucker and also into the oral cavity. The glands produce a granular proteinaceous secretion which stains strongly with the Mallory and Mazia methods, but gives no reaction for esterases or phosphatases. The distribution of the ducts conveying the secretion to the exterior indicates that it is probably used in adhesion.

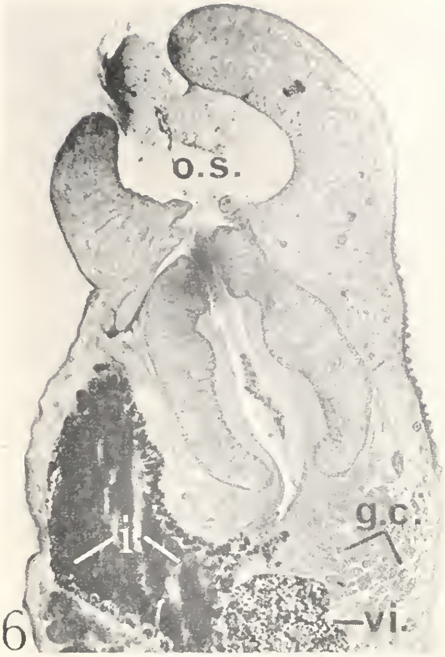
The oral cavity is linked with the large muscular and bulbous pharynx by means of a short cuticle-lined buccal tube, into which the anterior portion of the pharynx projects. The wall of the pharynx contains, in addition to muscular elements, a number of large cells with prominent nuclei and nucleoli, and a series of small vacuoles of unknown function ranged along the inner and outer surfaces at regular intervals (Fig. 6).

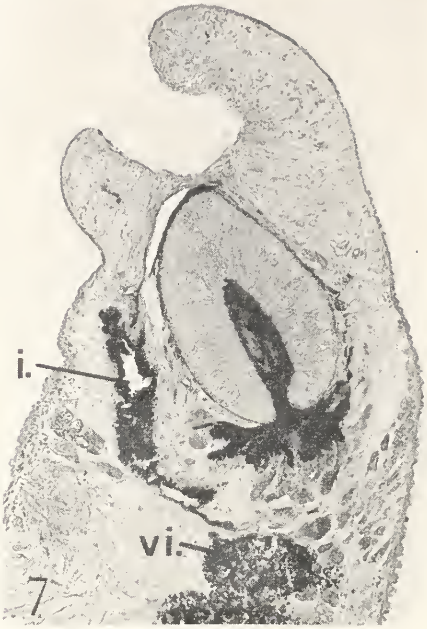
The pharynx leads *via* the esophagus into a bifid intestine whose ceca run the length of the body and give off branches which in turn repeatedly subdivide and anastomose. The esophagus is a short muscular tube surrounded by numerous unicellular glands arranged in two distinct zones. The cells of the inner zone, immediately around the esophagus, are smaller and produce a granular secretion giving an intensely positive reaction for alkaline phosphatase, while the outer larger cells produce a more coarsely granular and strongly acidophilic secretion quite free of phosphatases (Fig. 7). Fresh frozen sections and aqueous extracts of the esophageal region rapidly cause cavitation in gelatine films, indicating the production of a proteolytic enzyme by these esophageal glands, but it was impossible to determine which type of gland cell was responsible.

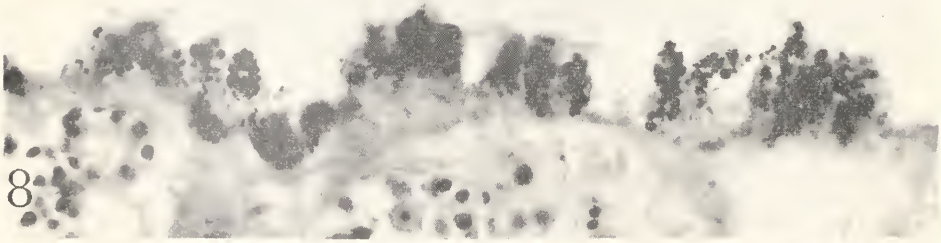
Both types of gland cell discharge through long unbranched ducts which enter the pharynx at its posterior end and run forward between the cuticular lining and the underlying musculature to open finally into the anterior end of the pharynx lumen (Figs. 6 and 7).

During feeding the oral sucker is flattened and flared against the bladder wall, and contraction of radial muscles within the sucker draws up a plug of bladder tissue whose tip reaches the anterior end of the pharynx. The plug is held secure by the constricting grip of the oral sphincter around its base while the sucking action of the pharynx, aided no doubt by proteolytic secretions from the esophageal glands, ruptures capillaries and draws blood into the intestine. Bladder tissue is never ingested, however, and very little damage is caused to the bladder epithelium.

The structure of the gastrodermis in *Polystoma* has been described elsewhere (Jennings, 1959). In brief, the gastrodermis is a single-layered discontinuous structure made up of columnar cells 16–18 μ tall and 8–10 μ wide, with basal vesicular nuclei and cytoplasm containing varying amounts of the pigment hematin, interspersed with areas devoid of cells and where only a thin basement membrane separates the gut lumen from underlying body tissues (Fig. 8). The hematin is contained within spherical vesicles up to 8 μ in diameter and the number of these increases with age, so that a mature cell is loaded with pigment and the nucleus obscured. When this condition is reached the vesicles are extruded into the gut lumen or, more commonly, the entire cell disintegrates either *in situ* or after being shed from the gastrodermis. The vesicles themselves persist intact for some time, but eventually rupture to discharge their contained hematin. New, younger cells







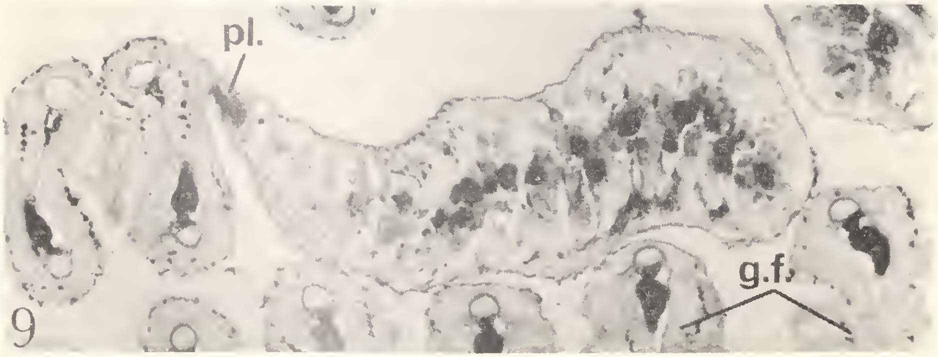


FIGURE 6. *Polystoma integerrimum.* Longitudinal section of the anterior region. g. c., gland cells posterior to the pharynx whose ducts run forward between the inner cuticular lining and musculature of the pharynx and open in its anterior portion; i., intestine, lined by a dis-

grow up to replace the spent cells and fill in the gaps in the gastrodermis, and thus the latter structure is in a state of constant degeneration and renewal.

Digestion in *Polystoma* occurs by a combination of extracellular and intracellular processes. Erythrocytes entering the intestine are immediately hemolyzed and within three hours of ingestion their nuclei have also disintegrated. The freed nuclear material mixes with the other gut contents and causes the whole mass to stain lightly with Feulgen, but this reaction eventually disappears as digestion progresses.

The intraluminar phase of digestion is accompanied by absorption of semidigested substances by the smaller younger cells of the gastrodermis, and their cytoplasm becomes swollen with spherical aggregations of material showing the same staining reactions as that remaining in the gut lumen. These cells show intense alkaline phosphatase activity along their distal margins and this is obviously concerned with the process of absorption. The enzyme is best visualized by the azo-dye method since the black cobalt-sulphide end product of the calcium-salt technique may be masked by any hematin present.

Absorption from the gut lumen continues until no stainable material remains. This situation is reached 24–48 hours after a meal, depending upon the amount of blood ingested. Digestion is completed intracellularly in the vesicles within which material is aggregated as it is absorbed from the lumen, but of the enzymes concerned in the process, only non-specific esterases could be demonstrated histochemically. These are localized within the vesicles and cannot be demonstrated in the cytoplasm of the gastrodermal cells.

As intracellular digestion proceeds, stainable material disappears from the vesicles and is replaced by granules of hematin resulting from degradation of the hemoglobin content of the meal. The hematin remains within the cell and thus the amount seen in a mature cell about to be shed from the gastrodermis probably represents an accumulation from the digestion of several meals.

Extrusion of hematin vesicles or the shedding of intact spent cells occurs 24–48 hours after a meal and consequently there is at this time a marked increase in the amount of hematin lying free in the gut lumen. Many of the freed vesicles, prior to rupturing, still show traces of esterase activity and this confirms a suggestion made in an earlier account (Jennings, 1959) that enzymes concerned primarily with intracellular digestion may remain in the hematin vesicles and be eventually transported to the gut lumen where they are released when the vesicles rupture. Due to the ramifications of the gut in *Polystoma* there is never complete evacuation between meals, and it is likely that these enzymes of intracellular origin will still be

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continuous pigmented gastrodermis and containing hematin granules mixed with heavily staining hemolyzed erythrocytes; o. s., oral sucker containing material regurgitated from the intestine; vi., vitellaria. Mallory. Scale: 1 cm. = 75 μ.

FIGURE 7. *Polystoma integerrimum.* Longitudinal section of the anterior region, showing intense alkaline phosphatase activity in the inner zone of gland cells associated with the pharynx. Abbreviations as in Figure 6. Gomori azo-dye method. Scale: 1 cm. = 75 μ.

FIGURE 8. *Polystoma integerrimum.* Transverse section through the gastrodermis, showing the discontinuous structure and the intracellular aggregations of hematin. Mallory. Scale: 1 cm. = 20 μ.

FIGURE 9. *Diplozoon paradoxum.* Longitudinal section of an individual fixed *in situ* on the host gill. g. f., gill filament; pl., plug of gill tissue drawn up and held by the buccal sucker. P.A.S. Scale: 1 cm. = 250 μ.

present in the lumen when the next meal is taken, and thus contribute to the intraluminar digestive phase.

No specific source of the intraluminar digestive enzymes was located, other than the hematin vesicles, and it seems likely, therefore, that the proteolytic secretions of the esophageal glands will play an important part in extracellular digestion, entering the intestine with the food and initiating hemolysis and nuclear breakdown.

The principal endproduct of hemoglobin digestion in *Polystoma* is hematin but a small proportion of the hemoglobin is converted to hematoidin, an iron-free, acid-soluble crystalline substance closely related to the bile pigments. Hematoidin crystals are only rarely found in histological preparations of *Polystoma,* however, due to their solubility in the standard fixatives, but can be seen in fresh squash preparations of the gastrodermis in about 10% of the cells.

2. *Diplozoon paradoxum*

Diplozoon paradoxum feeds predominantly on blood, but small amounts of gill tissue, epithelial cells and mucus are also found amongst the gut contents.

The adult *Diplozoon* consists of two individuals united in permanent copulation, with organic fusion of their bodies midway along the long axis, so that the composite individual is X-shaped. Each individual retains a terminal ventral mouth opening into a buccal cavity which bears laterally a pair of buccal suckers. An oval, muscular pharynx, devoid of glandular elements, protrudes slightly into the buccal cavity and leads backwards into the intestine. This extends posteriorly in each individual as a single much-branched cecum, and where the bodies of the two individuals fuse, the two ceca unite by a median canal, so that the two intestines are confluent.

Diplozoon lives attached to the gills of the host minnow by the clamps of the two opisthaptors and during feeding one or both of the anterior ends attaches itself to a gill filament by means of the buccal suckers. The grip is aided by adhesive secretions produced by clusters of gland cells around the buccal cavity which open on to the anterior body surface. The buccal suckers draw up a plug of gill tissue (Fig. 9), in much the same manner as the oral sucker in *Polystoma* draws up a plug of bladder tissue. The plug extends through the buccal cavity to the pharynx, which is protruded slightly and applied to the tip. Prolonged suction bursts the superficial blood capillaries, and blood, together with a small amount of gill tissue, enters the intestine. There is no evidence indicating the use of histolytic secretions to effect rupture of the gill capillaries, and no serious damage is caused to the gill filaments by the feeding activities of the trematode.

The gastrodermis resembles that of *Polystoma* in that it is a discontinuous and deciduous structure whose individual cells contain the characteristic hematin-laden vesicles. The cells are interspersed with areas of basement membrane either devoid of cells or covered by thin, extremely flattened and unpigmented young cells.

The course of digestion follows closely that observed in *Polystoma,* hemolysis of the erythrocytes occurring during or very soon after ingestion and being followed by partial intraluminar digestion. Soluble substances are absorbed by the gastrodermis and digestion subsequently completed intracellularly, with the production of hematin as a visible insoluble endproduct. As in *Polystoma* the cells actively absorbing materials from the gut lumen show intense alkaline phosphatase activity distally and this decreases as the cell ages and reduces its digestive functions. No esterase reaction could be demonstrated in the gastrodermis, but the entire nervous system shows intense cholinesterase activity and this provides a simple but effective means of demonstrating the system *in toto* (Halton and Jennings, 1964).

The vitelline glands of the reproductive system are in intimate contact with the intestine for most of its length and show at all times positive reactions for alkaline phosphatase, lipase and aminopeptidase, indicating metabolic activity possibly concerned with absorption and utilization of food materials from the gastrodermis.

In a few instances the intestine of newly fed *Diplozoon* contained, in addition to the hemolyzed blood, a number of reddish needle-shaped crystals 150–200 μ in length. These were water-soluble but could be fixed in absolute ethyl or methyl alcohol, when they stained strongly with the benzidine technique for hemoglobin. The crystals gradually disappeared in the living animal as digestion progressed, and they probably resulted from crystallization of hemoglobin released from hemolyzed erythrocytes and concentrated by absorption of water from the gut contents during the early stages of digestion.

3. *Discocotyle sagittata*

Discocotyle sagittata appears to feed exclusively on blood drawn from the superficial capillaries of the trout gills.

The mouth is anterior and ventral, and opens into a buccal cavity possessing laterally a pair of very large bilobed buccal suckers. The buccal cavity opens posteriorly into a small muscular non-glandular pharynx.

It was not possible to observe *Discocotyle* in the act of feeding, but judging from the similarities in structure and habit it is likely that the breaching of the host capillaries and the ingestion of blood are effected in the same manner as in *Diplozoon*. The buccal suckers are larger and more powerful than in the latter species, however, while the pharynx is relatively smaller, so that the suckers probably play a greater part in creating the necessary suction. No evidence of the production or use of proteolytic secretions could be found, and no significant amount of damage is caused to the gill filaments by the feeding activities of the trematode.

Neither gill tissues nor mucus were observed in the gut contents of the specimens examined.

The pharynx opens directly into the bifid intestine whose ceca extend to the posterior end of the body and give off numerous lateral branches which in turn subdivide and ramify between the vitellaria and other organs.

The gastrodermis resembles that of *Polystoma* and *Diplozoon,* and is made up of large hematin-laden cells, 18–20 μ long and 4–6 μ tall, which are interspersed with smaller, flattened non-pigmented cells and areas completely devoid of cellular elements.

The appearance of the gastrodermis indicated that digestion in *Discocotyle* follows much the same course as in *Polystoma* and *Diplozoon,* and this was confirmed from histological examination of individuals fixed at progressive intervals after removal from the host. Hemolysis and intraluminar digestion are accompanied by active absorption of the products by the gastrodermis, with subsequent completion of digestion and production of hematin within intracellular vesicles. Absorption is particularly noticeable in the smaller non-pigmented cells, and both these and the larger cells show intense distal alkaline phosphatase activity.

Hematin is eliminated from the gastrodermis by extrusion of the vesicles or by the sloughing off of intact spent cells.

It was not possible to demonstrate the presence of proteolytic enzymes in the gastrodermis, by histochemical methods, but as in *Diplozoon* the vitellaria give strong positive reactions for lipase and aminopeptidase.

4. *Diclidophora merlangi*

Diclidophora merlangi feeds chiefly upon blood but small amounts of gill tissue and mucus are also ingested.

The mouth is ventral and subterminal, and opens into a typical buccal cavity with lateral paired buccal suckers. The pharynx is spherical, muscular and devoid of glandular elements, and feeding is effected by suction of the host tissue.

A long esophagus links the pharynx with the bifid intestine whose ceca give off lateral much-branched diverticula. The latter are enveloped by the numerous vitellaria of the reproductive system.

The gastrodermis in *Diclidophora*, as in the other Polyopisthocotylea already described, is a discontinuous and deciduous structure whose cells contain varying amounts of hematin and show the characteristic distal zone of alkaline phosphatase activity. The cells are much smaller than in the other genera investigated, however, and even when fully mature and loaded with hematin are only 6–8 μ long and 3–4 μ tall.

Digestion in *Diclidophora* is effected by a combination of extra- and intracellular processes and follows much the same course as in *Polystoma,* except that hematin appears to be the sole endproduct of hemoglobin degradation and no traces of hematoidin were found. A small amount of non-specific esterase activity can usually be demonstrated in the gut contents of specimens fixed soon after feeding, but this does not increase in amount with time and appears, in fact, to be derived from the gill tissue ingested along with mucus as the subsidiary component of the diet. In control sections of whiting gill approximately 10% of the epithelial cells showed non-specific esterase activity and it is likely that the activity seen in the *Diclidophora* gut contents originates in these cells.

The gastrodermal cells show no enzyme activity, other than alkaline phosphatase, that could be detected by the techniques used, but the vitellaria, as in the other genera studied, give positive reactions for lipase and aminopeptidase.

5. *Octodactylus palmata*

Octodactylus palmata feeds predominantly on blood drawn from the host gill capillaries but as in *Diclidophora* and *Diplozoon*, this diet is supplemented by gill tissue and mucus.

The terminal mouth opens into a buccal cavity which possesses a pair of large lateral buccal suckers. Gill tissue is drawn up through the mouth, and suction by the bulbous and highly muscular pharynx ruptures the capillaries and draws blood into the intestine. The pharynx is devoid of gland cells and its action in procuring the food appears to be entirely mechanical.

The gut contents of recently fed *Octodactylus* generally include mucus and gill tissue in somewhat larger quantities than are found in *Diclidophora* and *Diplozoon,* but no appreciable damage to the gill filaments of the host was observed.

The intestine is of the usual polyopisthocotylean type, being bifid with the ceca of considerable length and giving off many branched lateral diverticula.

The gastrodermis differs somewhat from that of the other genera examined in that only relatively few areas are completely devoid of cells at any one time, and these are usually restricted to the walls of the two main ceca. The cells are small, as in *Diclidophora,* and range from 3–8 μ in height and 6–8 μ in width. The great majority of the cells contain hematin but the pigment granules are generally all confined within a single large vesicle, 3–6 μ in diameter, rather than distributed amongst four or five smaller vesicles as, for example, in *Polystoma.* The larger-sized vesicles often fill the entire cell and displace the nucleus to one side away from its normal basal position. In fixed preparations the vesicles appear as solid masses of hematin, but in fresh squashes the individual pigment granules are free and exhibit constant Brownian movement within the confines of the vesicle.

Digestion in *Octodactylus* follows the pattern observed in the other polyopisthocotyleans studied. Hemolysis is completed very soon after ingestion and then intraluminar digestion is accompanied by absorption and the completion of digestion intracellularly. The gastrodermal cells show a distal zone of high alkaline phosphatase activity which is particularly intensified during absorption.

The hematin resulting from intracellular degradation of hemoglobin accumulates within the vesicles until eventually the distal margins of the individual cells break down and the hematin is discharged into the gut lumen. During this process, and while the cell is recovering, the cell becomes crescent- or cup-shaped and the disorganized distal margin shows only diffuse alkaline phosphatase activity. Cells in this condition may continue to absorb material from the lumen, however, and often show a single small secondary hematin vesicle basally. This increases in size as the cell recovers from expulsion of the primary vesicle and moves distally, almost fills the cell, and is eventually expelled.

The gut contents in *Octodactylus* often show non-specific esterase activity but, as in *Diclidophora,* there is every indication that this originates in the gill tissue and not in the gastrodermis. No other enzymatic activity could be demonstrated histochemically in the intestine, but again lipase and aminopeptidase were abundant in the vitellaria.

6. *Plectanocotyle gurnardi*

None of the specimens of *Plectanocotyle gurnardi* available for examination was recently fed, but since the gastrodermal cells contain at all times large amounts of hematin it is concluded that blood forms the dominant, if not the sole, component of the diet. No traces of gill tissue or mucus were found, but this could conceivably be due to the progress of digestion since the previous meal.

The alimentary system resembles that of *Diclidophora* or *Diplozoon,* with paired buccal suckers, a muscular pharynx and a bifid, much-branched intestine. The gastrodermis is of the typical discontinuous and deciduous type, with somewhat sickle-shaped pigmented cells interspersed with naked areas devoid of cells.

Discussion

These observations on the nutrition of a number of monogenetic trematodes confirm indications available from previous accounts that there is a fundamental difference between the Monopisthocotylea and Polyopisthocotylea as regards the dominant components of the diet. The three monopisthocotyleans studied, from quite different parasitic locations, all feed on the host's epidermis and epidermal secretions, and similar feeding habits have been described in *Entobdella squamata* (Heath, 1902), *Megalocotyle marginata* (Folda, 1928), *Leptocotyle minor* and *Acanthocotyle* sp. (Llewellyn, 1954), *Entobdella soleae, Capsala martinieri, Trochopus* sp. and *Acanthocotyle* sp. (Kearn, 1963). Thus, this type of diet would appear to be a characteristic feature of the Monopisthocotylea. Uspenskaya (1962), however, states that in four other species (*Dactylogyrus vastator, D. solidus, Anchylodiscoides parasiluri* and *Tetraonchus monenteron*) varying amounts of blood are found in the intestinal contents, together with gill tissue and mucus, but the latter substances predominate.

In the Polyopisthocotylea, in marked contrast, blood forms the major, and sometimes the only, component of the diet. Of the species examined in the present study, *Polystoma integerrimum* and possibly *Discocotyle sagittata* feed entirely upon the host's blood, while *Diplozoon paradoxum, Diclidophora merlangi* and *Octodactylus palmata* supplement the blood diet with varying quantities of gill tissue and mucus. Ingestion of blood, or the presence of an intestinal pigment which is presumably hematin and hence indicative of a blood diet, has also been reported in *Hexacotyle* sp., *Onchocotyle* sp., *Octocotyle* sp. and *Microcotyle* sp. (Goto, 1895); *Axine* spp., and *Diclidophora* spp. (Goto, 1895; Llewellyn, 1954); the larval and neotenic adult stages, as well as the normal adult stage, of *Polystoma integerrimum* (Gallien, 1934; Llewellyn, 1954); *Kuhnia scombri* (Sproston, 1945; Llewellyn, 1954); *Hexabothrium appendiculata* and *Anthocotyle merlucci* (Llewellyn, 1954) and *Pricea cybium* and *Protomicrocotyle caranx* (Uspenskaya, 1962).

It is reasonable to suppose that the earliest Monogenea lived ectocommensally upon fish in much the same sort of way as modern Tennocephalida live on crustacean and other hosts. The fish epidermis and its mucoid secretions would form a readily available and rapidly replenished source of food to the flatworm, once the association was established, and by utilizing this the primitive Monogenea would become truly ectoparasitic. On this view the modern monopisthocotylean Monogenea, living as a rule upon the external surface of the host, retain ancestral feeding habits and the only modification found is the evolution in groups such as the entobdellid species of a specific feeding mechanism involving the use of histolytic "salivary" secretions. Even species such as *Calicotyle* which have sought the shelter of the host's cloaca, and are apparently on the way to becoming endoparasitic, still use the original type of food.

The polyopisthocotylean Monogenea, on the other hand, are predominantly gill parasites, having migrated into the branchial chamber of their piscine hosts, and they have departed considerably from the supposedly primitive feeding habits. The highly vascularized gill filaments offer an extremely nutritious and, again, readily available food in the form of blood, and the basic monogenean feeding mechanism of a suctorial pharynx is capable of obtaining this food without any modification other than slight elaboration of the oral and buccal sucker system. Thus, the differences in diet between the Monopisthocotylea and Polyopisthocotylea have not affected the feeding mechanism, and the anterior region of the alimentary system remains remarkably constant in structure throughout the Monogenea and, indeed, the Digenea. This uniformity contrasts sharply with the situation in the Turbellaria, where considerable diversification in the form of the pharynx is linked with the utilization of a wide variety of prey, ranging from Protozoa and many other invertebrates to tunicates (Jennings, 1957).

The differences in diet within the Monogenea do, however, have considerable effect upon the cellular structure of the gastrodermis. In the Monopisthocotylea digestion of the food creates no particular problem as regards the elimination of unwanted endproducts, even though the process is completed intracellularly. In the Polyopisthocotylea, however, the diet of blood and the retention of the intracellular digestive phase result in the intracellular production of hematin. The elimination of this insoluble substance is achieved at the expense of the continuity of the intestinal lining, and produces the discontinuous or *deciduous* gastrodermis characteristic of the sub-order. This involves wastage of cellular materials and thus the Polyopisthocotylea appear to be incompletely adapted to a blood diet. A more complete adaptation would be the extracellular formation of hematin, or the degradation of hemoglobin along some other pathway which allows the unwanted iron to be eliminated in a soluble form. The Trematoda are in fact capable of evolving such digestive processes, and these are seen in certain sanguinivorous Digenea such as *Haematoloechus* and *Haplometra* (Halton, unpublished work).

A compensating factor arising from the disintegration of gastrodermal cells in the Polyopisthocotylea is that intracellular enzymes are released to mingle with the gut contents and initiate breakdown of the next meal. Unfortunately it has proved impossible to localize the source of other digestive enzymes in either the Monopisthocotylea or the Polyopisthocotylea with the techniques at present available. It seems likely that the secretions of esophageal glands, poured on to the food during ingestion, play an important part in the extracellular phase of digestion, but this cannot be conclusively demonstrated. Certainly, however, the gastrodermis in the Monogenea has not evolved to the point of specialization of cellular function, for it shows no signs whatsoever of differentiation into glandular and absorptive or phagocytic components. In this respect it differs radically from the gastrodermis of other members of the phylum Platyhelminthes, such as the triclad Turbellaria, and of other acoelomates, such as the Rhynchocoela, where well differentiated gland cells occur and where there is separation of secretory and absorptive or phagocytic functions (Jennings, 1962a; 1962b).

SUMMARY

1. A comparative study has been made of the food, feeding mechanism, gut structure and digestive processes in representatives of the two sub-orders of the Trematoda Monogenea.

2. The two sub-orders differ fundamentally as regards the dominant components of the diet, the Monopisthocotylea feeding on the epidermis and associated mucoid secretions of the host while the Polyopisthocotylea feed primarily upon the host's blood. In some instances the Polyopisthocotylea supplement the diet with small amounts of host tissue and mucus.

3. The feeding mechanism in both groups consists basically of a muscular pharynx, and ingestion is the result of muscular suction, aided in some cases by histolytic secretions produced in pharyngeal or esophageal glands and used to erode the host tissues.

4. The two sub-orders differ considerably with regard to the structure of the gastrodermis, that of the Monogenea being a continuous cellular structure as in most other animals while in the Polyopisthocotylea it is a discontinuous and deciduous structure whose cells contain varying amounts of the pigment hematin.

5. Digestion in both the Monopisthocotylea and Polyopisthocotylea is effected by a combination of extra- and intracellular processes, but in the Polyopisthocotylea intracellular degradation of hemoglobin results in the accumulation within the gastrodermal cells of insoluble hematin, and the elimination of this substance results in the deciduous gastrodermis characteristic of the sub-order.

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DIGESTIVE ENZYMES OF THE CRYSTALLINE STYLE OF STROMBUS GIGAS LINNE.[1] I. CELLULASE AND SOME OTHER CARBOHYDRASES

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The crystalline style is a flexible hyaline rod composed of mucoprotein gel. Among marine molluscs it occurs in most lamellibranchs and in a few gastropods. The digestive enzymes of the crystalline style are set free into the gut lumen by dissolution of the style as it is rotated against a cuticular gastric shield by the cilia of the style sac epithelium. Yonge (1932) pointed out that *Strombus gigas,* feeding on fine filamentous algae, was the largest of herbivorous gastropods. Yonge's observations were later confirmed by Robertson (1961) and by Randall (1964), who showed that this conch feeds unselectively on delicate macroscopic algae, on unicellular algae, and on algal detritus. Microscopic examination of the stomach contents of *Strombus gigas* Linné from the Miami area has confirmed the ability of this animal to digest cell walls of filamentous green algae and to dissolve algal cytoplasm. These changes were particularly marked in green algae adherent to the surface of the head of the crystalline style.

A cellulase enzyme system would facilitate use of algal cellulose by *S. gigas.* Cellulolytic activity has been demonstrated in the crystalline style of the clams, *Mya* and *Mactra* (Lavine, 1946), an oyster, *Ostrea* and a mussel, *Mytilus* (Newell, 1953), an African bivalve, *Caelatura* and a marine snail, *Melanoides* (Fish, 1955), the wood-boring pelecypods, *Bankia* (Nair, 1955, 1957) and *Teredo* (Greenfield and Lane, 1953), and the lamellibranchs, *Cardium* and *Scorbicularia* (Stone and Morton, 1958). During feeding experiments Dean (1958) observed that algal cells of *Cryptomonas* were destroyed while this alga was swimming near the style of an oyster, *Crassostrea.* In this paper the cellulase activity of the crystalline style of *S. gigas* will be described. Subsequent papers in this series will examine other enzymes of the crystalline style.

MATERIALS AND METHODS

Specimens of the queen conch, *S. gigas,* which is native to Southeast Florida and the West Indies, were collected from shallow water adjacent to Virginia Key, Miami, Florida. They were maintained in sea water pens on the laboratory grounds until they were used. The style of *S. gigas* is large; one of the largest in the present series of samples measured 18.5 cm. long, 0.55 cm. wide, and weighed

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2.100 gm. The average moisture content was 82.4% and N averaged 8.19% by a micro-Kjeldahl method. Broken algal fragments and other detritus are often embedded in the substance of the portion of the style (*ca.* 0.5 cm. in length) protruding from the style sac into the lumen of the gut.

The animals were separated from the shell and the style removed quickly. The exposed head of the style was cut off and discarded. The surface of the remainder of the style was scraped with a sharp knife to remove adherent debris. It was then washed by shaking several times in sterile sea water. The styles were then homogenized in a sterile Waring Blendor with sterile 0.66 *M* phosphate buffer (pH 6.55) made isotonic with sea water by the addition of NaCl. This medium was 0.620 *M* NaCl, the same as that used earlier for the extraction of cellulase in *Teredo* (Greenfield and Lane, 1953). The viscous homogenate was stored at 4° C. for 12 hours, then centrifuged at 20.200 *g* at 4° C. for 30 minutes. The small residue was discarded. The supernatant solution was lyophilized, and the resulting powder was stored at − 10° C. There was no significant loss of cellulolytic activity during three months of storage. The powder was dissolved in citric acid-sodium phosphate buffer at room temperature and centrifuged briefly at low speed. The supernatant solution was filtered through a Millipore membrane. The sterility of the resulting enzyme solution was established by broth culture with Difco Tryptic Soy Broth. Cultures were negative for growth at both 24 and 48 hours at 30° C. Control experiments employed the sterile enzyme solution that had been boiled for 10 minutes.

Cellulolytic activity was estimated both by the formation of reducing sugar and viscosimetrically. Reducing sugar was estimated by the methods of Somogyi (1928, 1952) as modified by Nelson (1944). Optical density was measured at 500 mμ with either the Beckman Model DU spectrophotometer or Coleman Model 6A spectrophotometer. Samples of sodium carboxymethyl cellulose (CMC) from Hercules Powder Company, of different degrees of polymerization (D.P.) were used as substrates in the cellulase assay. Purified sodium alginate (Fisher Scientific Company), and carrageenan (Marine Colloids, Inc.) were also employed. In testing for cellobiase, glucose was estimated by the Glucostat Special reagent (Worthington Biochemical Corp.). The pH of the reaction mixture was determined with a Beckman glass electrode before and after incubation. Digestive activity was expressed as μg of reducing sugar liberated per milligram of dry style extract per hour.

Changes in substrate viscosity were measured in Ostwald viscosimeters. Three ml. of substrate solution (CMC to final concentration 0.8%) were mixed in the viscosimeter with 3.0 ml. of McIlvaine's citric acid-sodium phosphate buffer (pH 6.75). After thermal equilibrium in the 35° C. water bath had been achieved, the time required to empty the capillary was noted. One-tenth ml. of enzyme solution was added and mixed in a stream of air bubbles. The time required to empty the capillary was measured at intervals from the time of mixing.

RESULTS

Filtration through Millipore membranes does not affect the activity of style enzyme solutions as judged by two different criteria (Tables I and II). The yields of reducing sugar from CMC 70 of three different degrees of polymerization in-

TABLE I

*Hydrolytic activity of crystalline style extract before and after bacterial filtration**

Substrate	Unfiltered enzyme solution	Millipore-filtered enzyme solution	Boiled enzyme solution
CMC 70 M	90.5 ± 9.0	89.7 ± 10.3	11.7 ± 1.7
Phosphoric acid-swollen cellulose	10.3 ± 0.7	10.9 ± 1.1	3.8 ± 0.4
Cellulose powder	2.1 ± 0.3	1.9 ± 0.3	0.7 ± 0.2
Sodium alginate	5.7 ± 0.3	6.1 ± 0.5	0.6 ± 0.3
Carrageenan	2.4 ± 0.7	2.7 ± 0.5	0.7 ± 0.2
Cellobiose	8.9 ± 0.5	8.7 ± 0.5	3.7 ± 0.4

* Activity expressed as μg. reducing sugar/mg. crystalline style powder/hour.
Each value is the mean and standard deviation of 10–17 determinations.
Reactants incubated at 35–37° C. at pH 6.75.

cubated with crystalline style enzyme solutions are shown in Table III. This table shows that different degrees of substitution in the CMC substrate had only a slight effect on the activity of the enzyme preparation. These results differ somewhat from those reported for *Limnoria* by Ray (1959). In this form maximum cellulase activity was found when CMC 70 High was the substrate. When the cellulase activity of *S. gigas* style extract was estimated viscosimetrically, however, the results, shown in Figure 1, more nearly resemble those reported by Ray. Whatman No. 1 filter paper, swollen in 85% phosphoric acid and suspended at 0.5% concentration in phosphate buffer and the same concentration of Whatman cellulose powder, was also used as a substrate. These results also appear in Table I. The style enzyme solution is less effective on these substrates than on the CMC samples used. Figure 2 shows the linear relationship between cellulase activity and enzyme concentration measured by protein nitrogen. The relationship between pH and cellulolytic activity is shown in Figure 3. There is a single peak of activity between pH 6.8 and pH 7.2. When determined viscosimetrically after 60 minutes incubation the pH optimum was 6.75. The pH of gut contents of *S. gigas* ranged from 6.25 to 6.65.

TABLE II

Viscosity of enzyme-substrate before and after bacterial filtration*

Substrate		Incubation time				
		15 min.	30 min.	60 min.	90 min.	120 min.
CMC 70 M	I	67.8 ± 1.4	54.7 ± 1.2	44.9 ± 2.0	39.6 ± 2.2	36.5 ± 2.4
	II	74.2 ± 3.1	62.9 ± 3.2	52.1 ± 3.3	47.4 ± 2.7	44.5 ± 1.8
Sodium alginate	I	89.0 ± 3.2	80.9 ± 2.8	67.7 ± 2.1	58.2 ± 2.9	52.3 ± 2.8
	II	88.1 ± 2.2	82.2 ± 3.8	72.0 ± 3.2	63.9 ± 3.2	56.7 ± 2.7
Carrageenan	I	90.9 ± 1.9	83.3 ± 2.5	74.7 ± 2.2	68.4 ± 1.8	64.3 ± 2.2
	II	90.7 ± 5.8	86.9 ± 0.1	79.8 ± 0.6	74.2 ± 0.4	70.7 ± 0.1

* Viscosity is expressed as time in seconds for capillary emptying.
I = Unfiltered enzyme solution.
II = Millipore-filtered enzyme solution.

TABLE III

Cellulase activity of the crystalline style of Strombus gigas with various substrates

Substrate	D.S.	Viscosity	µg. glucose/mg. of enzyme/hr.
CMC 70 low	0.82	30 cps.	31.4
CMC 70 med.	0.77	470 cps.	41.1
CMC 70 high	0.72	2100 cps.	15.0
CMC 90 high	0.94	280 cps.	17.8
CMC 120 high	1.25	135 cps.	7.8
Swollen filter paper			3.2
Cellulose powder			0.7

Incubation at 30° and pH 7.4 for CMC, pH 7.3 for other substrates.

The optimum temperature and the temperature of inactivation of the enzyme solution were determined. The relationship between the incubation temperature and cellulase activity showing an optimum about 40° C. is presented in Figure 4. Thermal stability of the enzyme solution was determined by assaying residual activity after heating the enzyme solution to various temperatures for fifteen minutes

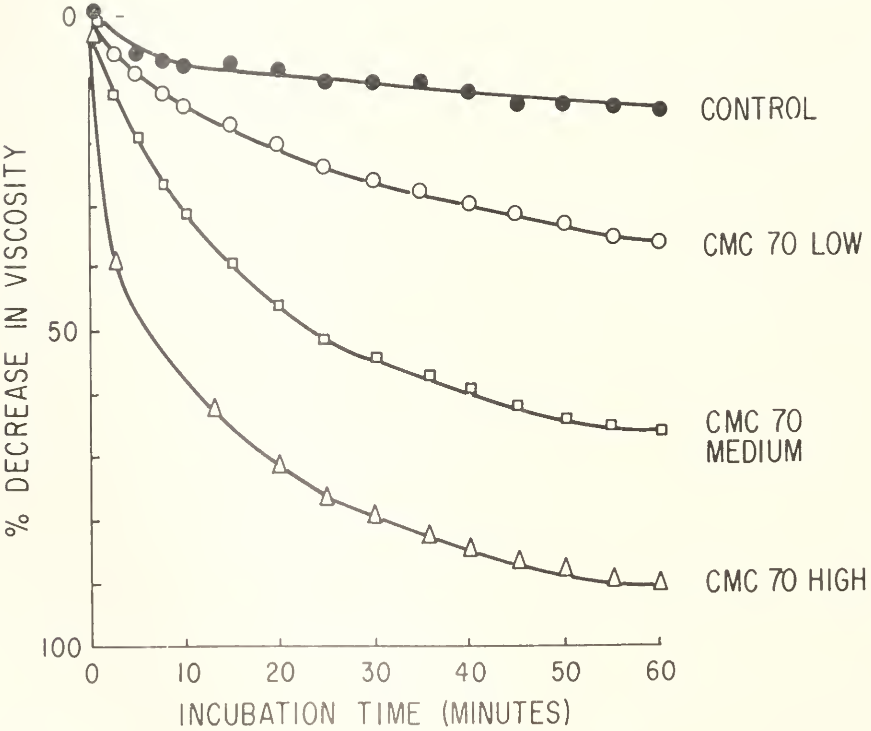

FIGURE 1. Effect of crystalline style enzyme on CMC 70 of different viscosity. Final concentration of CMC 70 was 0.8%; pH was 6.75 and temperature 35° C.

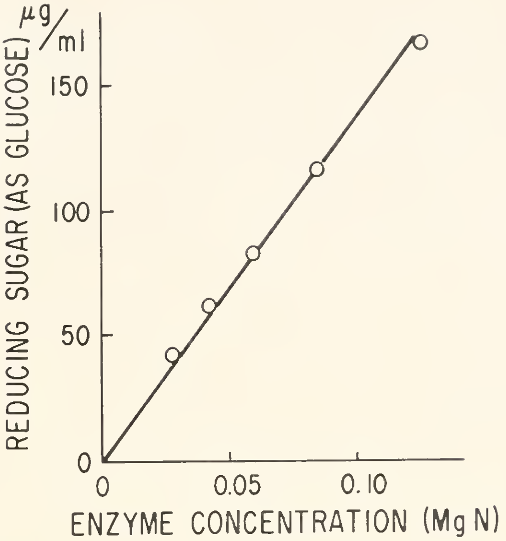

Figure 2. Cellulase activity of crystalline style extract at various enzyme concentrations. Reaction mixtures containing 12 mg. CMC 70 of medium viscosity were incubated at 30° C., pH 6.8 for two hours.

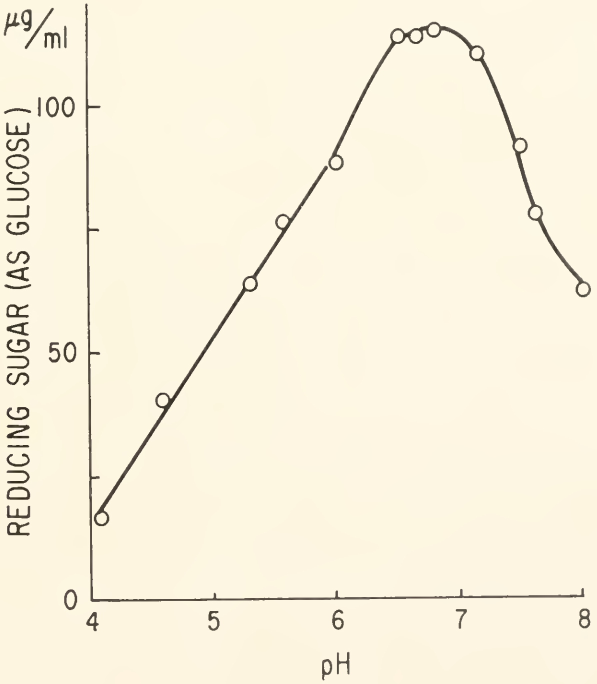

Figure 3. Cellulase activity of the crystalline style at various pH levels. Substrate was CMC 70 of medium viscosity; temperature was 30° C.; time was two hours.

(Fig. 5). Activity was undiminished up to 45° C. Between 45° C. and 50° C., cellulolytic activity was markedly reduced and was 90% destroyed at 70° C.

Cellobiase activity (Table IV) was estimated by incubating cellobiose with crystalline style enzyme solution. Glucose produced was determined by the specific glucose oxidase method (Worthington Biochemical Corp.). As compared with the cellulase activity, the cellobiase activity is slight. Since the crystalline style of *S. gigas* appears to be deficient in cellobiase, it appears that digestion of cellulose by this animal does not necessarily include cellobiose as an intermediate (Levinson and Reese, 1950).

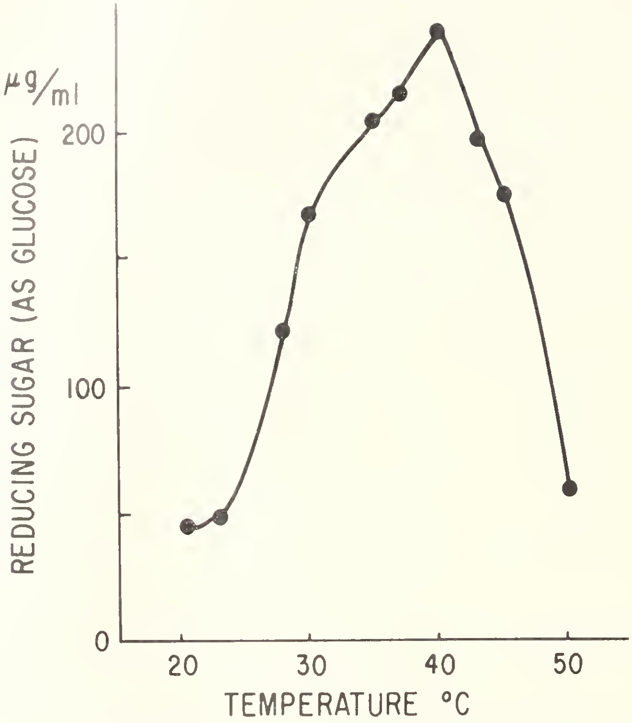

FIGURE 4. Effect of temperature on cellulolytic activity of the crystalline style extract. Substrate was CMC 70 medium; pH 6.75; incubation time was two hours.

The relationship between decreasing viscosity and increasing concentrations of reducing sugar during cellulolysis is presented in Figure 6.

DISCUSSION

There is general reluctance to attribute cellulase enzyme activity to higher Metazoa because symbiotic microorganisms are involved in cellulose breakdown in most higher animals. Indeed, Morton (1952, 1960), Newell (1953) and Barrington (1962) have emphasized the occurrence of spirochaetes in the crystalline style of many bivalves. Some, at least, of the enzymatic capability of the style is attributed to these symbionts.

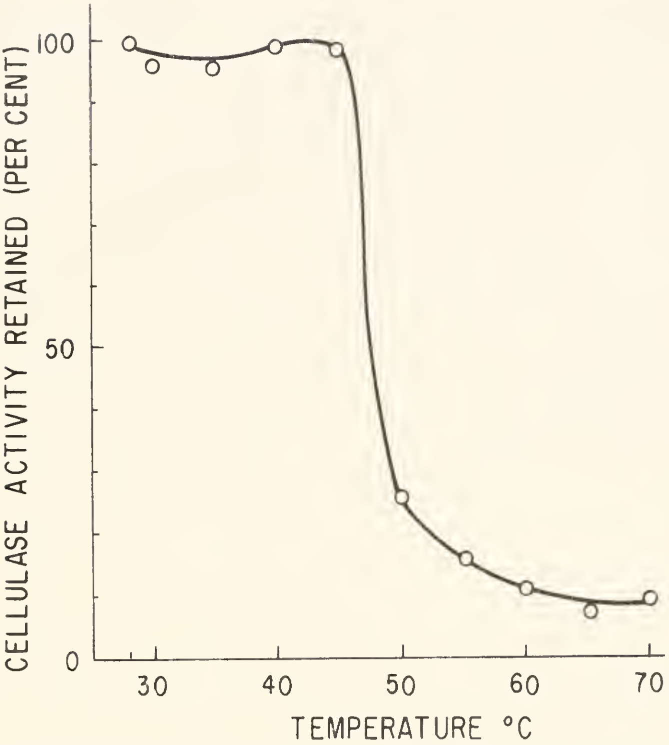

FIGURE 5. Temperature stability of cellulase in the crystalline style. Activity of the heated enzyme, assayed after two hours in a reaction mixture containing CMC 70 medium, at 35° C., pH 6.8.

Levinson and Reese (1950) suggested that at least two kinds of enzymes were involved in the complete degradation of native cellulose. First, a C_1 enzyme causes a rapid decrease in viscosity by converting native cellulose to linear anhydroglucose chains. These are then hydrolyzed to the soluble sugars glucose and cellobiose by C_x enzymes (Gascoigne and Gascoigne, 1960; Levinson and Reese, 1950). Our results strongly suggest that the cellulolytic capability of the crystalline style of *S. gigas,* together with certain other amylolytic activities, are of molluscan rather than bacterial origin. If it be assumed that CMC in solution is a straight-chain molecule made up of 1,4-β-glucose linkages (Levinson and Reese, 1950), then all the

TABLE IV

Cellobiase activity of the crystalline style of Strombus gigas Linné

pH	5.7	6.6	7.6
Glucose (μg.)	16.3	16.2	10.3

0.5% cellobiose in McIlvaine buffer was incubated at 35° C. for three hours. Reaction mixtures contained 1.8 mg. of lyophilized style.

soluble derivatives of cellulose used in this study were hydrolyzed by a C_x enzyme of the crystalline style. Digestion of algal cellulose by *S. gigas* probably includes some preliminary microbiological degradation followed by extracellular digestion by style enzymes in the stomach (Evans and Jones, 1962). Digestion of lower molecular weight sugars and other partially digested foods is probably completed intracellularly in phagocytic cells of the digestive diverticula.

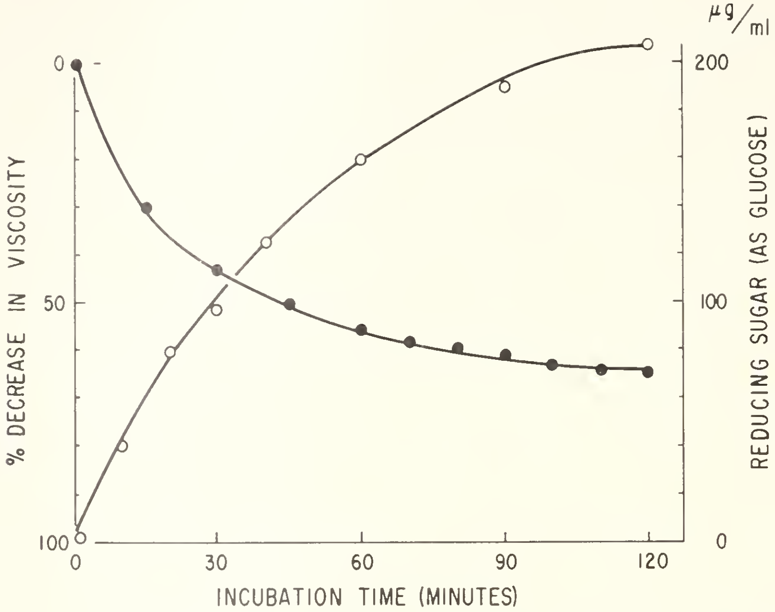

FIGURE 6. The relationship between decrease in viscosity and formation of reducing sugar during cellulolysis by the crystalline style. Reaction mixture contained 0.6% CMC 70 medium viscosity; pH 6.75 at 35° C.

Summary

The crystalline style was extracted in buffered saline and the extract subsequently lyophilized. The activity of the resulting enzyme powder was determined by measuring the amount of reducing sugar it liberated from various substrates under different conditions, and by measuring the decrease in viscosity of these substrates. Cellulase activity was proportional to enzyme concentration. The pH optimum was between pH 6.8 and pH 7.2. Optimum temperature for enzyme activity was 40° C. Between 45 and 50° C., cellulolytic activity was markedly reduced. Cellobiase activity of the style extract was slight. Bacteria-free extracts were as active as unsterile preparations. Some implications of these observations are discussed.

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THE HEMOCYTES OF RHODNIUS PROLIXUS STAL.

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Wigglesworth (1933) classified four kinds of hemocytes in *Rhodnius* nymphs, and in 1955 he identified two additional types. His interest centered on the most abundant hemocyte, which will be referred to in the present paper as the *plasmatocyte*. He (1956a, page 142) stated that plasmatocytes ". . . show definite signs of secretory activity just at the time when hormone of the thoracic gland is being produced," and he (1956b, page 97) concluded that one of the distinct functions of the plasmatocytes is that ". . . in the early stages of moulting they seem to play some essential part in the production of the moulting hormone by the thoracic glands." Wigglesworth (1956b) demonstrated that the plasmatocytes possess mucopolysaccharide inclusions and mentioned that this material was liberated during the later stages of moulting. The signs of secretory activity which Wigglesworth stated occur in the plasmatocytes of fourth stage nymphs were an average increase in cell size and the sudden appearance of many, clear, non-staining vacuoles in the cytoplasm between the third and fourth day after these insects took a blood meal.

The initial purpose of these studies was to examine quantitatively these two criteria for secretory activity in the circulating plasmatocytes of *Rhodnius* in fresh, unfixed, unstained coverslipped samples of hemolymph with dark phase contrast microscopy. As these studies were being made, it became evident from the form and behavior of the different types of hemocytes that a change in the terminology of the different cells was needed.

Hemocytes were examined daily throughout the fourth and fifth stages and in adults at 970 to 1400 ×. The hemocytes were also studied using supravital methods and in fixed and stained smears. Hemolymph was obtained from a severed leg or antenna.

RESULTS

1. *General observations*

The hemolymph of *Rhodnius* is a clear, pale, straw-yellow, watery fluid. When examined in a hanging-drop preparation or in a moist chamber the hemolymph does not obviously coagulate or gel in 24 hours, although a very finely granular precipitate may form on long standing as the drop partially darkens. In a very few cases, a rare plasmal veil may be observed. In a moist chamber, the hemolymph slowly darkens and finally appears either pale brown or has an irregular scattering of dark sooty patches, but the drop never becomes generally dark brown or uniformly black *in vitro*. None of the hemocytes darken or blacken.

2. *Phase contrast observations and classification*

Using the classification and terminology suggested by Jones (1962), the various types of hemocytes of *Rhodnius* can be readily identified in fresh, undiluted, unfixed, unstained hemolymph with a dark phase contrast microscope.

a. *Prohemocytes*

The prohemocytes are always small, mostly round to ovoid cells, generally with a relatively large, single, centrally-located, round nucleus (Plate I, Figs. 1 and 2). Usually the nucleus has extremely fine, dark grey, granular chromatin material around a single, slightly excentric round nucleolus. Prohemocytes have a relatively small amount of smooth, dark grey, homogeneous, or sometimes finely granular cytoplasm. In some cases, the nucleus may be excentric and ovoid, with a single slight indentation; the nucleolus may be irregular in shape, or, in a few cases, absent. Prohemocytes can be seen with a few, relatively large, dark grey, round inclusions. On a number of occasions, prohemocytes have been seen to degenerate *in vitro*: the small, round, cartwheel-like nucleus is generally ejected, and the cytoplasm rounds up into a pale-grey sphere with fine dancing particles within and around it (Plate I, Fig. 3). On many occasions, a prohemocyte has been seen to undergo unmistakable and intense ameboid movements *in vitro,* when none of the other hemocytes made comparable movements. During such ameboid movements, the nucleus was frequently constricted or otherwise distorted. In older nymphs, the prohemocytes measured from 5 to 7 microns in diameter. Mitotic divisions were seen *only* in cells slightly larger than the typical prohemocyte. At metaphase, the chromosomes were so tightly packed that they appeared as a single dark-grey bar. The metaphase plate was contained in a clear, hyaline central zone within the cell. Vacuoles and granules were usually conspicuous in dividing cells. Prophases could not be accurately identified in the preparations examined.

b. *Plasmatocytes*

Plasmatocytes are exceedingly variable in form (Plate I, Figs. 5, 6, 9, 10–15). The most common variety is an ovoid cell with a single, large, centrally-located, round-to-ovoid nucleus containing a single, round-to-ovoid nucleolus. The cytoplasm contains sharply-outlined round, ovoid, or short rod-shaped, or tear-drop-shaped granular inclusions (Plate I, Figs. 5, 6, 14, 15). With dark phase microscopy, the edges of these inclusions are generally sharp black and the enclosed granular space bright.

In unfixed plasmatocytes, the cytoplasm often contains few (two) to many (about 32) round or irregular, clear, non-refringent, watery, colorless vacuoles of various sizes (Plate I, Figs. 11–13). Some plasmatocytes can be seen with large, less sharply-defined, round or spherical, grey inclusions, often having a very pale greenish cast.

Plasmatocytes tend to send out several to many, fine, thread-like pseudopodia (Plate I, Figs. 5, 11–13). In some cases, exceedingly thin cytoplasm will spread out from the cells and terminate in extremely delicate spikes. Round, spindle, and irregular forms of plasmatocytes abound in the hemolymph. Small, medium and large varieties of each of the above may be found in a single preparation.

Although the plasmatocytes have a distinct tendency to spread out and form peripheral thread-like pseudopodia in thin wet films, they were very rarely observed to perform vigorous ameboid movements comparable to those seen in some prohemocytes. Although the plasmatocytes are probably capable of ameboid activity *in vivo,* such movements do not critically distinguish plasmatocytes from other types of hemocytes.

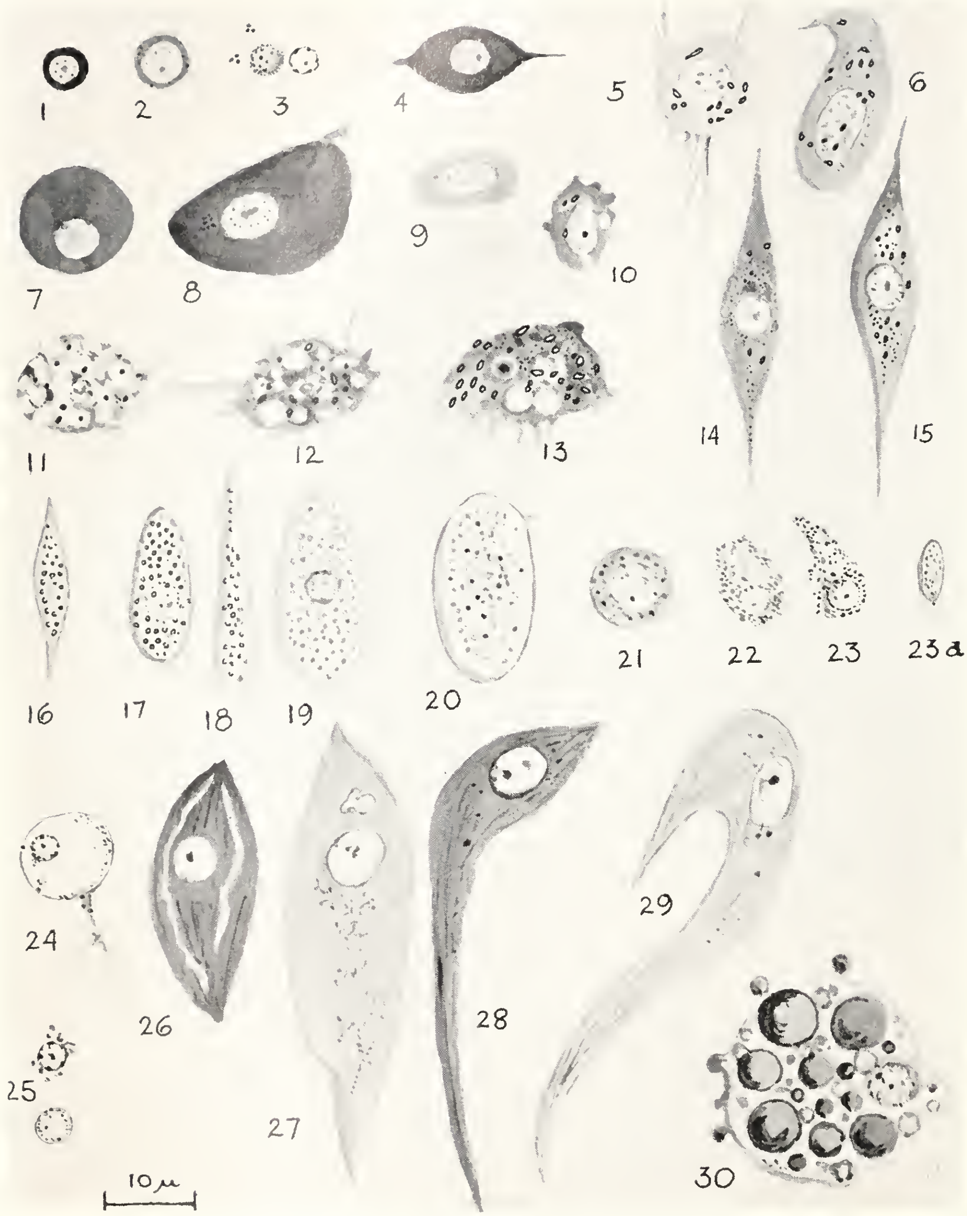
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PLATE I

Phase contrast appearance of *Rhodnius hemocytes*.

FIGURES 1 AND 2. Typical prohemocytes.

FIGURE 3. Lysed prohemocyte. The nucleus is at the right and the cytoplasmic envelope and granules at the left.

FIGURE 4. Small spindle oenocytoid with excentric nucleus and without inclusions.

FIGURE 5. Non-vacuolated plasmatocyte with thread-like pseudopodia.

The sizes of plasmatocytes throughout the fourth stadium of *Rhodnius* were noted but were so highly variable in both unfixed and methanol-fixed films within and between individuals that no difference in average sizes could be detected at any time during the stage with the methods used. Since many more hemocytes are available in the fifth stadium, plasmatocytes were measured every day between the first through ninth, eleventh through sixteenth, and on the twentieth days after nymphal feeding. Plasmatocytes in unfed fifth stage nymphs and in unfed adults were also measured. In all, 335 plasmatocytes were measured. The minimum values for individual widths varied from 4.4 to 8.8 microns and the maximum values from 8.8 to 17.6. The minimum values for individual lengths ranged from 7.7 to 11 and the maximum values from 12.1 to 66 microns. The mean widths of the plasmatocytes during the fifth stadium varied from 7.7 to 12.2 and the lengths from 9.9 to 20.6 microns. The overall mean dimensions of the plasmatocytes for the period examined were 9.8 (standard error 0.3) by 14.5 (standard error 0.6) microns. There was a distinct tendency for the plasmatocytes to decrease in length during the fifth stadium while the widths did not change greatly. Measurements were made on a series of coded slides, but the writer was unable to accurately distinguish between plasmatocyte sizes at any time during the fifth stadium.

Although Wigglesworth (1955) stated that the average size of the plasmatocytes increased between the third and fourth days after fourth stage nymphs feed, measurements made on the cells shown in his Figure 5 A and B show statistically insignificant differences in mean width, length, circumference and area (Table I). The same applies to the plasmatocytes illustrated in his Figure 6 (Table I). But what is striking about Wigglesworth's Figures 5 and 6 is that the fixed adherent plasmatocytes of fourth stage nymphs are strikingly and significantly smaller than the unfixed circulating forms (Table I). Whether this is due to fixation or to the spreading-out of plasmatocytes in the fresh preparation is not clear. The fixed adherent plasmatocytes illustrated in Wigglesworth's Figure 6 average about the

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FIGURE 6. Non-vacuolated plasmatocyte without pseudopodia.

FIGURES 7 AND 8. Round and ovoid oenocytoids with characteristically excentric nucleus and with dark, smooth cytoplasm.

FIGURE 9. Small plasmatocyte without inclusions, vacuoles, or pseudopodia.

FIGURE 10. Small plasmatocyte with a few inclusions, vacuoles, and blunt pseudopodia.

FIGURES 11–13. Typical vacuolated plasmatocytes with obscured nucleus and large watery vacuoles and discrete inclusions.

FIGURES 14–15. Spindle plasmatocytes showing very fine granules (mitochondria?) and larger inclusions (mucopolysaccharide?).

FIGURE 16. Spindle form of granular hemocyte.

FIGURES 17–19. Typical ovoid granular hemocytes.

FIGURE 20. Large granular hemocyte with one, short blunt extrusion at upper right.

FIGURE 21. Round granular hemocyte.

FIGURES 22–23. Partially lysing granular hemocytes.

FIGURE 23a. Very small intact granular hemocyte with obscured nucleus.

FIGURE 24. Lysing granular hemocyte, showing the hyaline cytoplasm and round cartwheel-like nucleus.

FIGURE 25. Fully lysed granular hemocyte.

FIGURE 26. Large oenocytoid with glassy rod-like inclusions.

FIGURE 27. Large oenocytoid with finely granular network and one vacuole.

FIGURES 28–29. Typical large oenocytoids with characteristic excentric nucleus and long threadlike filamentous inclusions.

FIGURE 30. Adipohemocyte with excentric nucleus and various sizes of fat-like droplets.

same as the unfixed circulating forms measured in the present study. It is concluded from the present observations and from calculations on Wigglesworth's figures (Table I), that a change in the sizes of plasmatocytes is not a practical criterion for their possible secretory activity because of an inherently large individual variation.

In fresh wet films, various inclusions in many plasmatocytes were observed to very rapidly turn into clear, colorless vacuoles. Unfixed circulating plasmatocytes of 72 fourth stage *Rhodnius* were classified as either with or entirely without vacuoles in differential counts of generally 100 hemocytes per insect per group of two to five nymphs, two to five days after ecdysis, and daily after the nymphs fed, for a period of 12 days. Each insect was used only once. During the fourth stage the mean percentages of plasmatocytes in fresh hemolymph varied from 22.7 to 61.5, with an overall mean of 44.2. Of these, 83.8% to 96.8% (mean of 92.3%, standard error of 0.8) had few-to-many vacuoles in their cytoplasm. Even in unfed nymphs, 94.3% of the plasmatocytes encountered in differential hemocyte counts were vacuolated. A few extremely vacuolated plasmatocytes were seen in only four insects during the fourth stadium. It is evident from these findings that vacuolation of circulating plasmatocytes cannot be a very useful criterion of changes in their possible secretory activity during the fourth stage. It is not clear why there should be such a great discrepancy between the present findings and those of Wigglesworth (1955). Wigglesworth's Figure 5 shows very few vacuoles on the third day and a considerable (about 10-fold) increase in vacuoles on the fourth day in circulating plasmatocytes. His Figure 6 indicates that fixed adherent plasmatocytes have many more vacuoles than the unfixed circulating forms on the fourth day.

TABLE I

Calculations made from plasmatocytes of fourth stage Rhodnius, as illustrated by Wigglesworth (1955), with standard errors

Figure	No. measurements	No. cells	Days after nymphal feeding	Mean width (μ)	Mean length (μ)	Mean circumference (μ)	Mean area (μ²)
5A	1	4	3	12.4±0.6	29.6±2.9	91.8±14.9	156.8±19.8
	2			11.6±0.4	27.8±2.8	98.8±17.9	157.8±26.6
5B	1	3	4	13.7±0.9	36.0±2.9	127.0±20.0	186.2±36.6
	2			13.0±1.3	34.4±2.5	132.7±14.2	185.6±30.0
6A	1	7	3	8.3±0.3	16.2±1.0	33.8± 1.4	66.9± 5.7
	2			8.2±0.2	16.5±1.0	38.6± 1.7	66.7± 5.7
6B	1	6	4	10.1±0.8	16.0±1.1	26.6± 2.6	108.3±17.0
	2			10.4±0.9	16.1±1.1	39.9± 2.9	105·3± 6.7

Unfixed circulating plasmatocytes of fifth stage *Rhodnius* were sub-divided into those with no or very few vacuoles and those with few to many vacuoles in differential counts of generally 200 hemocytes per insect per group of four to five nymphs. Thirty-nine insects were used for the first seven days after ecdysis, a separate group being examined each day. One hundred and one nymphs were used to study changes following the blood meal, generally five insects being studied daily from the day the nymphs took a blood meal to the twentieth day thereafter.

During the first seven days after ecdysis to the fifth stage, plasmatocytes in differential hemocyte counts varied from 47.1 to 68.7, and 95.9% to 98.4% of them were vacuolated. On the day the insects fed and during the next day, differential counts of plasmatocytes averaged 44.7% to 53.8%, and of these 96.1% to 99.3% were vacuolated forms. Between the first and second days after the nymphs took blood, however, a spectacular shift in the presence of vacuolated plasmatocytes was recorded. From the second through the twentieth day, circulating plasmatocytes averaged 29.8% to 63.7% (overall mean of 51.5%). Between the first and second days after nymphal feeding, the percentage of plasmatocytes which were classified as vacuolated forms dropped from 96% to 40%, and thereafter more or less steadily declined to about 8% on the twentieth day (*i.e.*, before ecdysis to the adult stage).

When five newly-ecdysed fifth stage *Rhodnius* were submerged for one minute in water at 55° C., it was observed that most (97.5%) of the plasmatocytes still vacuolated *in vitro;* but when nymphs which had taken a blood meal were similarly heat-fixed, very few of their plasmatocytes vacuolated *in vitro.* Fifth stage nymphs were heat-fixed on seven representative days after the blood meal and hemocytes from three to five insects for each day were examined. In differential counts, the plasmatocytes were classified as those with few or no inclusions and those with conspicuous round, ovoid or irregular, phase-dark inclusions. Plasmatocytes averaged 32.7% to 49%, and 82.1% to 93.5% (mean of 89.6%) of them had inclusions.

Clearly the problem of vacuolation of plasmatocytes in *Rhodnius* needs further study. But, if one assumes that the secretory activity of plasmatocytes is indeed correlated with cytoplasmic vacuolation, as Wigglesworth (1955) has indicated, then the present data collected on unfixed hemolymph could be interpreted to mean (1) that the circulating plasmatocytes are highly secretory throughout the entire fourth stadium, and during the fasting period after ecdysis to the fifth stage, and (2) that there is a remarkable decrease in their secretory activity shortly after fifth stage nymphs take a blood meal. Additional studies are clearly needed before such interpretations can be accepted. The present data do not support the idea that circulating plasmatocytes suddenly become secretory during the time when the thoracic gland hormone is being produced in either the fourth or fifth stadia.

c. *Granular hemocytes*

The granular hemocytes of *Rhodnius* are typically larger and noticeably thicker than plasmatocytes in thin films of hemolymph examined with phase microscopy (Plate I, Figs. 16–20). However, very small granular hemocytes have been seen in fresh hemolymph films (Plate I, Fig. 23a). Freshly withdrawn granular hemocytes often have a very pale, yellowish-brown cast, are ovoid to spindle in shape, and are characteristically filled with numerous, discrete, round, granular inclusions of a mostly uniform size from 0.5 to 1 micron. The granules generally tend to obscure the relatively small, round, centrally-located nucleus in fresh material. With the ordinary bright field microscope, the granules in these unfixed cells appear quite vague and could easily be overlooked or mistaken for fine droplets (vacuoles).

Of the numerous granular hemocytes examined in this study (approximately 50,000), only 20 were seen with one, short, blunt, or spike-like clear pseudopodium.

On a few occasions granulocytes have been seen to retract very rapidly their spindle ends and round up. Unlike plasmatocytes, some of the granular hemocytes have been observed to rock back and forth very slightly *in situ*. Unlike plasmatocytes, they were never observed to send out pseudopodia *in vitro*.

In unfixed films of hemolymph, the granular hemocytes occur in two very distinct forms: those which remain intact for long periods *in vitro* (Plate I, Figs. 16–20) and those which suddenly degenerate or lyse generally shortly after withdrawal of the hemolymph sample (Plate I, Figs. 22–25). Many granular hemocytes of various sizes have been watched as they degenerate *in vitro*. The intact cell seems to twist suddenly, contract or constrict along its longitudinal axis; and the cell may then collapse like a balloon, releasing the round, cartwheel-like nucleus and many fine dancing granules (Plate I, Figs. 21–23). Many times, as the cell breaks down, the cytoplasmic envelope fragments into two or more spherical hyaline masses with some enclosing dancing granules, as well as with granules around the masses. The granules suddenly released from the disintegrating granulocyte are much more sharply outlined than in the intact cell and may have a very faint greenish cast. The granules and cytoplasmic fragments do not quickly vanish but tend to maintain their identity for a considerable time. The cytoplasmic fragments and extruded nuclei greatly complicate both differential and total hemocyte counts. The lysing or lysed granular hemocytes strikingly resemble cystocytes (but they do not lead to coagulation or gelation of the plasma).

Submersion of *Rhodnius* in a water bath at 55° C. for one minute generally did not reduce the percentages of granulocytes lysing *in vitro*, but collection of fresh hemolymph into 0.75% Versene largely prevented lysis of these cells. Collections of hemocytes in 1% to 3% Versene severely damaged the hemocytes: the surface of the cells appeared abnormally thickened.

Widths and lengths of granulocytes were measured in unfixed wet films of hemolymph from fifth stage nymphs on eleven representative days after they took a blood meal. Of the 130 granular hemocytes measured, the minimal values for individual widths ranged from 4.4 to 9.9 microns and the maximal values from 11 to 16.5; the minimal values for individual lengths ranged from 9.9 to 13.2 microns and the maximal values from 22 to 35.2. The mean dimensions of the granular hemocytes were 10.3 (standard error 0.3) by 18.8 (standard error 0.4) microns. There was no marked change or trend in the length-width measurements of granulocytes during the fifth stage after feeding.

d. *Oenocytoids*

The cells which will be termed the *oenocytoids* of *Rhodnius* occur in two distinct forms. In fresh hemolymph examined with phase microscopy, the first variety is a relatively small, round, ovoid cell with one or two sharp spindle ends and the cytoplasm is very smooth, dark grey, and homogeneous. The nucleus is sharply outlined, round and characteristically excentric (Plate I, Figs. 4, 7 and 8). The second, and most commonly encountered variety of oenocytoid is a very large plasmatocyte-like cell, often occurring as bizarre variations of the spindle form (Plate I, Figs. 26–29). These large cells are characterized by having extremely fine, long, phase-dark filaments at the spindle ends and by a large excentric nucleus, often with two nucleoli. The inclusions are sometimes in the form of an irregular finely granular network or appear as delicate, faintly outlined glassy rods which have a very faint greenish cast (Plate I, Fig. 26). The large bizarre oenocytoids often occur in clusters of two to four, and in some cases appear fused to each other On some occasions, irregular nuclei and apparent binucleate forms have been seen. Some of these cells have been seen to send out a few filamentous cytoplasmic extensions.

e. *Adipohemocytes*

On rare occasions, hemocytes with an excentric nucleus and many brilliant fat-like droplets of various sizes have been encountered in the hemolymph (Plate I, Fig. 30). These cells should be termed *adipohemocytes* only when they can be clearly distinguished from fat body cells. At various times typical large fat body cells can appear in the hemolymph (in some cases, at least, their appearance results from accidental dislodgement at the time of sampling). Adipohemocyte-like cells have been seen in fixed whole-mounts of thoracic glands. The scarcity and erratic occurrence of the small adipohemocytes in the hemolymph make it most impractical to include these cells with other hemocytes in most differential counts. Since Wigglesworth (1955) has indicated that all gradations between adipohemocytes and typical fat body cells may be found in *Rhodnius*, it is useful to place all circulating cells containing fat droplets in a special category where they may be listed separately.

f. *Granulocytophagous cells*

Unmistakable plasmatocytes have been seen engulfing a single intact granulocyte and/or the nucleus of the lysed form (Plate II, Figs. 31–34). In addition, very large, plasmatocyte-like cells, measuring 20 to 35 microns or more in diameter, have been observed with two to eight engulfed intact granular hemocytes or their nuclei (Plate II, Figs. 35–39). These large phagocytes tend to send out characteristically very extensive pseudopodia, which may extend 30 microns and more beyond the main portion of the cell (Plate II, Fig. 39). While the very large forms *may* be only a hypertrophied form of plasmatocyte, they are sufficiently distinctive in size and activity to be listed separately in differential counts, and for convenience will be termed *granulocytophagous cells.* Whether the large granulocytophagous cells are, in fact, giant plasmatocytes is by no means certain.

3. *Observations on supravital preparations*

To further characterize the different types of hematocytes, fresh drops of unfixed hemolymph from fifth stage nymphs were collected on slides previously coated with a thin, even, dry film of the following dyes: (a) neutral red, (b) phenol red, (c) congo red, (d) eosin Y, and (e) Janus green B. Wet coverslip films were examined with and without phase contrast.

Neutral red was picked up by the plasmatocytes and concentrated within yellow, orange, or red cytoplasmic inclusions. The granular hemocytes and oenocytoids did not encorporate neutral red. The nuclei and cytoplasmic fragments from the lysing or already lysed granulocytes also did not stain. None of the hemocytes encorporated phenol red or congo red. The nuclei of lysing and fully disintegrated granular hemocytes stained pale rose with eosin Y but the cytoplasm and granules did not stain. Other types of hemocytes did not definitely encorporate the eosin. With Janus green B, the granules within intact granular hemocytes quickly became a pale but distinct slate blue; those nuclei which were ejected from lysing granulocytes did not stain. Many vacuolated plasmatocytes had a dull slate-blue cast to them but the vacuoles did not stain. In some plasmatocytes brilliant sky-blue inclusions were visible. Other hemocyte types did not stain supravitally with Janus green B.

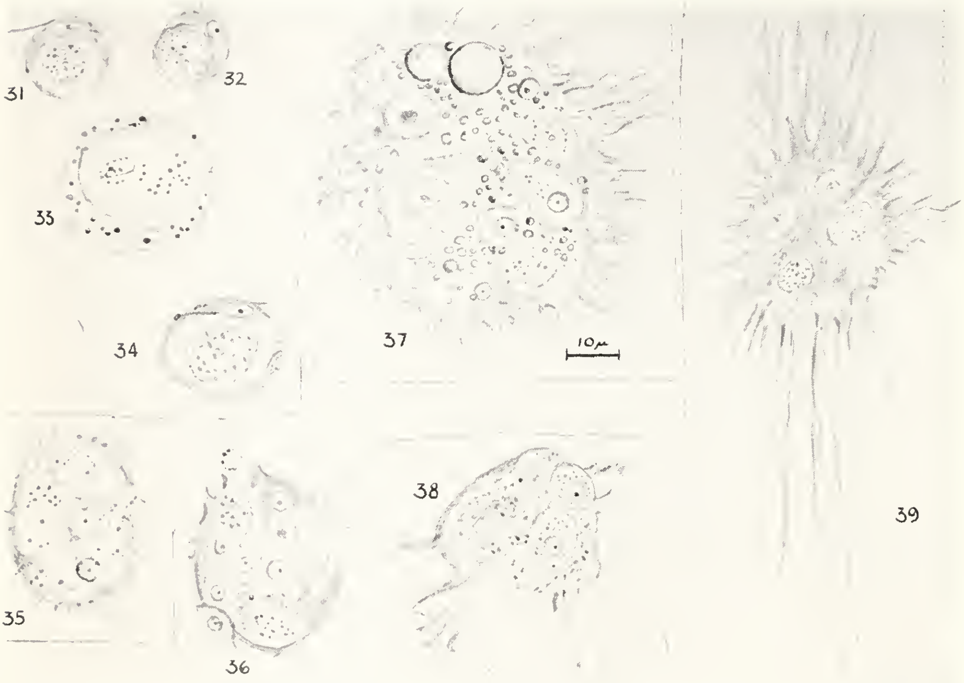

PLATE II

FIGURES 31–34. Plasmatocytes which have each engulfed a single granular hemocyte.

FIGURES 35–39. Large granulocytophagous cells which have engulfed two or more granular hemocytes and/or their nuclei. Note the very extensive pseudopodia in Figures 37–39.

4. *Observations on fixed and stained smears*

In thin and thick, air-dried smears of hemolymph obtained from unfixed or heat-fixed *Rhodnius* and subsequently treated with ethanol, methanol or Carnoy prior to staining with Giemsa or Wright stain or with Harris hematoxylin and eosin, the granular hemocytes generally could not be readily recognized, and none of the hemocytes appeared sharply acidophilic. In those granulocytes which were identifiable, the round inclusions did not stain but appeared as sharply outlined circular bodies, often with a faint yellowish cast against a pale blue or faint grey cytoplasm.

When the hemolymph was fixed with alcoholic Bouin and stained with eosin azur-II, those granulocytes which could be recognized had faintly pink-staining cytoplasm with colorless granules. The prohemocytes were varying shades of blue with faint red-purple nuclei. The oenocytoids were basophilic around the nucleus but the abrupt spindle ends of these cells were often distinctly eosinophilic. In some oenocytoids a fine purple-staining meshwork could be seen.

In general, regardless of the fixative employed, the different types of hemocytes did not stain sharply with vertebrate blood stains and were often very difficult to distinguish. Even the best stained smears were notably inferior to the examination of fresh unstained cells with phase contrast microscopy.

It is important to note, however, that Wigglesworth (1955) observed that the adherent granular hemocytes of *Rhodnius* were acidophilic after fixation with Carnoy or Bouin and staining with either Masson trichrome, hematoxylin and eosin, or with Prenant's ferric trihematin. In osmium-ethyl gallate preparations, Wigglesworth (personal communication) observed that the inclusions of the granular hemocytes appeared as colorless spheres or vesicles. In ordinary stained preparations, he found the granular hemocytes to be conspicuous for their lack of granularity.

Discussion

The terms used here for three of the hemocytes of *Rhodnius* present no problem relative to the terms used by Wigglesworth (1933, 1955) for the same cells; thus, the prohemocytes are equivalent to his proleucocytes, plasmatocytes are the same as his phagocytes or amebocytes, and the adipohemocytes are comparable to his adipoleucocytes or lipocytes. A complex problem arises, however, with the changes in the names of two clear-cut types of hemocytes: (1) the cells which are here termed the *granular hemocytes* of *Rhodnius* are referred to by Wigglesworth as *oenocytoids* and possibly also as large *granular cells*, and (2) the cells which are here termed *oenocytoids* are referred to by Wigglesworth as *large non-granular spindle cells* and as *non-phagocytic giant hemocytes* [Wigglesworth (personal communication) considers these to be variant forms of the plasmatocyte].

Wigglesworth (1933) considered the granular hemocytes to be comparable to the oenocytoids of Poyarkoff (1910) because (1) when fixed, the cytoplasm appeared homogeneous and stained with eosin, and (2) they were specifically phagocytized by certain other hemocytes. Nevertheless, the cells here termed *granular hemocytes* of *Rhodnius* differ strikingly from the oenocytoids of most other insects in that (1) the nucleus in intact cells is centrally located whereas in most oenocytoids the nucleus is characteristically excentric (Hollande, 1909, 1911; Poyarkoff, 1910; Bogojavlensky, 1932; Rooseboom, 1937; Yeager, 1945; Jones, 1962). (2) The granular hemocyte of *Rhodnius* is never a binucleated cell whereas oenocytoids may have two nuclei (Hollande, 1911; Bogojavlensky, 1932). (3) The cytoplasm of the granular hemocyte is not dense or elaborately textured whereas in most oenocytoids the cytoplasm is dense and contains canaliculi, threads or crystals (Bogojavlensky, 1932; Yeager, 1945; Nittono, 1960; Jones, 1962). (4) The granular hemocytes of *Rhodnius* stain with eosin in certain preparations (Wigglesworth, 1933), whereas the oenocytoids of many other insects are generally amphophilic or basophilic (Bogojavlensky, 1932; Yeager, 1945; Nittono, 1960; Jones, 1962), and at best are faintly eosinophilic (Yeager, 1945). (5) The granular hemocytes of *Rhodnius* are often very numerous in the hemolymph (they can make up 30% to 70% of the cells encountered in differential counts), whereas the oenocytoids have not been reported to rise even to the 10% level (Yeager, 1945; Jones, 1950; Nittono, 1960). (6) The granular hemocytes of *Rhodnius* do not pick up neutral red and thus differ from the *oenocytes* of several insects which are said to encorporate this dye; oenocytes are also said to stain supravitally with methylene blue, trypan blue, and Bismarck brown (Hollande, 1914; Poisson, 1924; Bogojavlensky, 1932; Koch, 1945; Ochse, 1946). (7) The granular hemocytes of *Rhodnius* do not possess the bizarre shapes of those oenocytes of *Rhodnius* illustrated by Wigglesworth (1953, his Fig. 239 g, h, and k), nor do granular hemocytes possess the spindle-shaped clefts or needle-like crystals which some of the oenocytes have (Wigglesworth's Fig. 239 e, f, h and i). It is important to mention, however, that Wigglesworth (personal communication) believes the granular hemocytes resemble certain small oenocytes in stained preparations of *Rhodnius* (see, for example, his Fig. 239 c and d).

With phase contrast microscopy, the granular hemocytes of *Rhodnius* resemble the non-phagocytic granulated blood cells of many other insects (Poisson, 1924, see his Fig. 21; Bogojavlensky, 1932; Millara, 1947; Jones, 1959 and unpublished). The granular hemocytes of *Rhodnius* do not closely resemble the oenocytoids of *Mysia* (Hollande, 1909), *Melolontha* (Hollande, 1911), *Psylliodes* (Hollande, 1911), *Galleria* (Metalnikov and Gaschen, 1922), *Notonecta* (Poisson, 1924), *Calliphora* (Rooseboom, 1937), *Prodenia* (Yeager, 1945), *Ephestia* (Arnold, 1952), *Tenebrio* (Jones, 1954), *Drosophila* (Rizki and Rizki, 1959, their "crystal cells"), *Bombyx* (Nittono, 1960), or *Galerucella* (Jones, unpublished). Wigglesworth (1933) stated clearly that the cells which we term *granular hemocytes* have no connection with the *oenocytes* of *Rhodnius*.

The cells which are termed the *oenocytoids* of *Rhodnius* (and which Wigglesworth considers to be a variety of plasmatocyte) have the following characteristics in common with the oenocytoids and/or oenocytes of many other insects: (1) they are generally large, thick, often quite bizarrely-shaped cells, (2) they have one or sometimes two nuclei with conspicuous nucleoli and a dense, often complexly textured cytoplasm, (3) they may occur in discrete clusters, and (4) they are not numerous in the circulating hemolymph. They differ from stationary oenocytes most conspicuously in not being eosinophilic cells. Whether the cells here identified as oenocytoids are related to or are derived from the stationary oenocytes of *Rhodnius* is not known. For a long time it has been claimed that oenocytes are capable of budding off certain hemocytes (Koschevnikov, 1900; Kollman, 1908; Hufnagel, 1918; Poisson, 1924) but generally the investigators do not clearly distinguish between small oenocytes, large plasmatocytes, granular hemocytes, or oenocytoids.

Several of the hemocytes of *Rhodnius* might be secretory (*e.g.*, plasmatocytes, granular hemocytes, and oenocytoids). Various cytological criteria are needed before we can accurately assess the situation. Total and differential hemocyte counts on *Rhodnius* will be presented in subsequent papers.

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Summary

1. The hemocytes of *Rhodnius prolixus* have been studied with phase contrast microscopy, after supravital staining, and in fixed and stained smears.

2. With phase contrast microscopy, the following categories of circulating cells can be readily identified: (a) non-dividing and mitotically-dividing prohemocytes, (b) non-vacuolated and vacuolated plasmatocytes, (c) intact and quickly lysing granular hemocytes, (d) oenocytoids with and without special cytoplasmic inclusions, (e) adipohemocytes, and fat body cells, and (f) granulocytophagous cells.

3. This classification and terminology are compared with those of Wigglesworth. It is suggested that the cell which Wigglesworth terms an *oenocytoid* is more comparable to the granulated blood cells of other insects and may be referred to as a granular hemocyte. It is suggested that the cells which Wigglesworth refers to as *large non-granular spindle cells* and *non-phagocytic giant hemocytes* are comparable to the oenocytoids of other insects.

4. Vacuolation of plasmatocytes can be prevented by heat-fixing fed *Rhodnius*. Lysis of granulocytes can be prevented by collecting hemolymph into 0.75% Versene.

5. Attempts to correlate an increase in sizes of circulating plasmatocytes with secretion of the thoracic gland hormone in fourth and fifth stage nymphs were not successful because of the great variability in the sizes of these cells.

6. Since most circulating plasmatocytes in differential hemocyte counts of unfixed fourth stage nymphs were identified as the vacuolated type, no correlation was possible between their vacuolation and the secretion of the thoracic gland hormone.

7. In unfed fifth stage nymphs, most of the circulating plasmatocytes were classified as vacuolated cells. Between the first and second days after the nymphs took a blood meal, the percentages of plasmatocytes identified as vacuolated cells abruptly decreased and steadily declined during the rest of the stadium.

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EVIDENCE FOR TRANSAMINASE ACTIVITY IN THE SLIME MOLD, DICTYOSTELIUM DISCOIDEUM RAPER[1]

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As the developmental cycle of the slime mold, *Dictyostelium discoideum* Raper, proceeds from the myxamoeba stage to the mature sorocarp stage, there occurs a reduction in proteinaceous materials and an increase in polysaccharide carbohydrate, as shown by Gregg, Hackney and Krivanek (1954) and Gregg and Bronsweig (1956). On the basis of these quantitative studies, Gregg and his associates suggested that the protein components may serve as precursors not only for energy-source intermediates of development, but also for the synthesis of carbohydrate necessary for stalk formation. Attempting to define the metabolic mechanisms responsible for this inverse relationship, Krivanek and Krivanek (1959) chromatographically analyzed the amino acid components of the slime mold in both hydrolyzed and unhydrolyzed tissue. Their findings suggested that deamination may be one process relating protein degradation to carbohydrate synthesis. These authors did not exclude the possibility, however, that other metabolic mechanisms, *e.g.*, transamination, may be instrumental in this linkage.

Utilizing a spectrophotometric technique to observe the change in the characteristic absorption band of DPNH at 340 mμ, Wright and Anderson (1959) demonstrated the occurrence of "aspartic-pyruvic transaminase." However, these authors, as well as others (Meister, 1950; Aspen and Meister, 1958), have expressed the lack of definity of such an analytical technique because of its broad specificity.

In view of the important role which transaminase activity plays in relating protein and carbohydrate metabolism, more precise evidence than that thus far presented would seem desirable. It is therefore the intent of this paper to demonstrate that specific transaminase activities are indeed operative in the slime mold, *Dictyostelium discoideum.*

MATERIALS AND METHODS

D. discoideum was cultured in the manner described by Bonner (1947), using *Escherichia coli* as the bacterial associate.

The following two transamination reactions were studied:

Reaction I: glutamate + pyruvate → α-ketoglutarate + l-alanine
Reaction II: glutamate + oxaloacetate → α-ketoglutarate + l-aspartate

The primary technique employed in studying these reactions was that of progressive chromatography as described by Hird and Rowsell (1950).

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Individuals in the desired stage of development were harvested from the Petri plates and homogenized in ice-cold phosphate buffer, pH 7.4, using an homogenizer of the type described by Gregg *et al.* (1954). The final volume of the homogenate was 5 ml. The extent of homogenization was determined by microscopic inspection of the homogenate.

After centrifugation of the homogenate, the supernatant or soluble fraction (S) was withdrawn from the particulate centrifugate or insoluble fraction (I). Both fractions were then made up to the original volume of 5 ml.

One-ml. samples of each fraction were then transferred to five separate reaction tubes—five tubes for each fraction series. To the control tube in each series was added 1 ml. of phosphate buffer only. To each of the remaining four tubes in each series were added ½ ml. glutamate and ½ ml. oxaloacetate or pyruvate, depending upon the reaction under consideration. The concentration of the glutamate, oxaloacetate and pyruvate varied and will be discussed under Results. To this point all steps were carried out under ice-cold conditions.

It is conceivable that l-alanine may be formed from pyruvate, l-aspartate from oxaloacetate, and α-ketoglutarate from glutamate by reactions other than transamination. Therefore, supplementary controls were utilized to determine whether the appearance and disappearance of the appropriate substrates were mutually interdependent. In this particular control series, only one of the initial reactants, *i.e.,* glutamate, was added to the reaction tubes, with subsequent treatment of these controls being the same as for the phosphate buffer controls and the experimental series.

Immediately upon the addition of the last substrate, the reaction tubes were put into a closed anaerobic environment, consisting of gaseous nitrogen and pyrogallol, and the mixtures were allowed to react at a temperature of 37° C. Reaction tubes from both soluble and insoluble series were generally removed after 30, 120, 180 and 240 minutes and processed. Control tubes were processed in the same manner and for the maximum time interval.

At the end of each incubation period, to each of the tubes were added two volumes of warm ethanol (60–70° C.) to precipitate the proteins. After centrifugation, the supernatant fluid was withdrawn and dried *in vacuo* at room temperature. The residue after evaporation was then resuspended in 1 ml. of distilled water, and identical fractions from each preparation were spotted on Whatman No. 1 filter paper for a uni-directional chromatographic separation of the amino acids. Among the various solvents used were: propanol-water (80:20), n-butanol-acetic acid-water (250:60:250), n-butanol-acetone-water (10:10:5), and n-butanol-acetone-water (5:4:1). Development of the spots was accomplished by means of spraying the chromatograms with a solution of 0.3% ninhydrin in 95% ethanol. Identification of the unknown spots was determined by means of positional comparisons between the unknown spots and spots of known amino acids applied to the same sheet.

The spot determination of the alpha-keto acids (oxaloacetate, alpha-ketoglutarate and pyruvate) was done by separating them as their 2,4-dinitrophenylhydrazones according to the method described by Block, Durrum and Zweig (1958). To samples of each of the reaction mixtures, after deproteinization with warm ethanol, was added 1 ml. of 0.5% 2,4-dinitrophenylhydrazine dissolved in 6 N HCl. After 30 minutes' standing at room temperature, the hydrazones were extracted in a separatory funnel with three $7\frac{1}{2}$-ml. washes of a chloroform:ethanol (80:20) solution. The hydrazones, now in the latter solution, were then extracted with $7\frac{1}{2}$ ml. of 1 N Na_2CO_3. After washing the hydrazone-containing Na_2CO_3 solution with 5 ml. of chloroform-ethanol solution, the Na_2CO_3 solution was then acidified with $2\frac{1}{2}$ ml. of 6 N HCl in the cold. The resultant acidified Na_2CO_3 solution was further washed with three portions of the chloroform:ethanol solution totaling 10 ml. The 10 ml. of washings were then evaporated under a gentle air stream.

For a chromatographic separation of the 2,4-dinitrophenylhydrazones, the residue after evaporation was dissolved in 2 ml. absolute ethanol and spotted on Whatman No. 1 filter paper in 1-μl. amounts. Identification of the spots was determined by preparing samples of known alpha-keto acids, processing them in the same manner as the experimental series and spotting them on the same paper with the experimentals.

Detection of the hydrazones of the keto acids was accomplished by initially inspecting the chromatogram for yellow spots (distinctive of hydrazones), then spraying the paper with a 2% ethanolic KOH solution which imparts a red-brown color to the spots, and/or scanning the paper with a UV light which caused the spots to fluoresce.

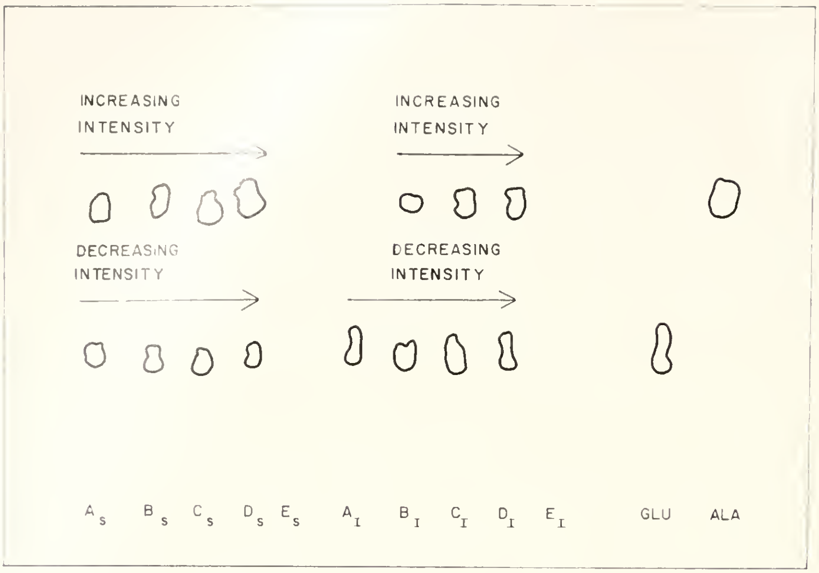
Since the paper chromatographic method did not afford a clear separation between glutamate and aspartate, a paper electrophoresis procedure was utilized (Block, Durrum and Zweig, 1958). This method is specific in its separation of aspartate, glutamate, histidine, arginine, lysine, and the monoamino-monocarboxylic acids. A phthalate buffer, pH 5.9, was used in a Spinco paper electrophoresis apparatus usually run at 500 volts, 18 amperes for three hours.

RESULTS AND DISCUSSION

A. Amino acids

Figure 1 shows the chromatographic results of Reaction I, *i.e.,* when tissue fractions, soluble and insoluble, were incubated in the presence of 1/40 *M* glutamate and 1/10 *M* pyruvate. It is to be noted that with lengthening periods of incubation (A is shortest, D is longest), color intensities of the glutamate spots decrease in both soluble and insoluble series. No corresponding spots were evident in the control series which were incubated for four hours—the maximum time for the experimental series. The appearance of alanine in the insoluble series lagged behind its appearance in the soluble series. Thus, after two hours' incubation alanine was first seen in the former series while only 30 minutes were necessary for it to appear in the soluble series.

The results relative to Reaction II are seen in Figures 2 and 3. In Figure 2, the spots of aspartate and glutamate in the soluble series are in close spatial relationship to each other, with aspartate trailing glutamate. The chromatographic technique did not clearly delineate the two compounds although development of the chromatogram with dicyclohexylamine did allow better interpretation than did ninhydrin. It is to be noted that aspartic acid increased in intensity in the soluble series. A corresponding decrease in the intensity of glutamate was also evident. In the insoluble series, no aspartate was apparent in either the chromatographic or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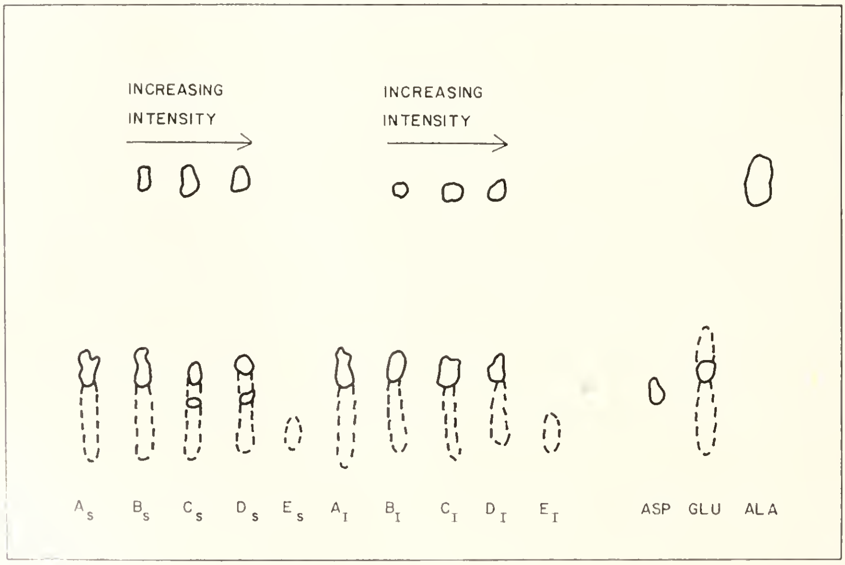
E_I
GLU
ALA

FIGURE 1. Exact reproduction of chromatogram showing amino acid results of Reaction I. Incubation times: A series, $\frac{1}{2}$ hour; B series, 2 hours; C series, 3 hours; D series, 4 hours; E series (phosphate control), 4 hours. "S" denotes soluble fraction, "I" denotes insoluble fraction, GLU = glutamate known; ALA = alanine known.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ASP
GLU
ALA

FIGURE 2. Exact reproduction of chromatogram showing amino acid results of Reaction II. Incubation times same as in Figure 1. ASP = aspartate known.

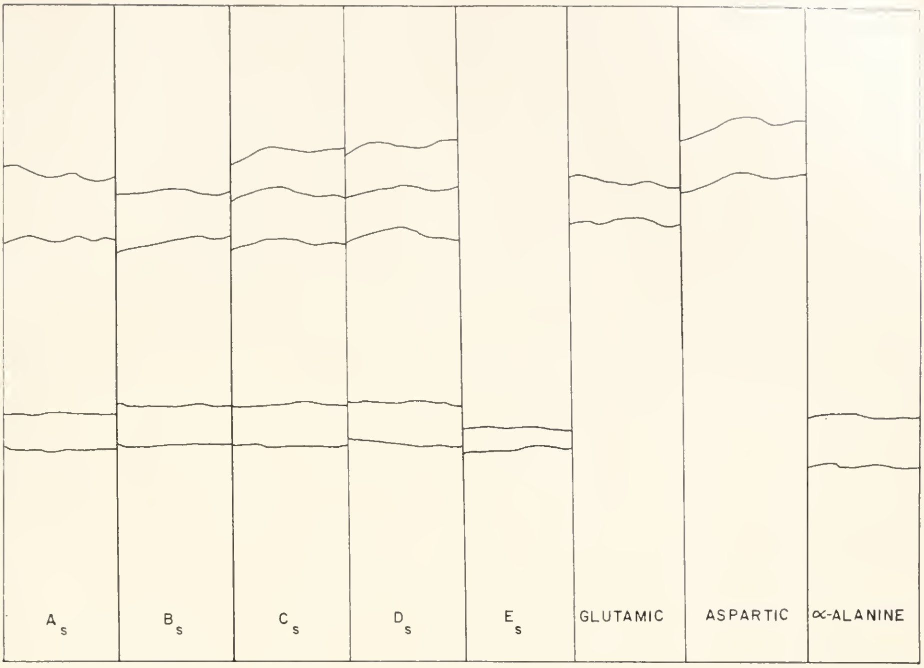

FIGURE 3. Exact reproduction of electrophoretic pattern showing amino acid results of Reaction II. Length of run: 3 hours; voltage: 500; amperage: 18 amps. Veronal buffer of pH 8.6. Letter notations same as in Figure 1.

electrophoretic determinations. It should be mentioned at this point that no decrease in color intensity of glutamate was observed when 1/20 *M* concentration was used. When 1/40 *M* glutamate was used, a perceptible decrease was evident. However, this decrease was less profound than in Reaction I.

In addition to the expected aspartate product of transamination Reaction II, an unexpected product—alanine—was also present in both soluble and insoluble series. No alanine was detected in the control series. The appearance of alanine could be accounted for by way of oxaloacetic acid being decarboxylated to pyruvic acid, with transamination subsequently occurring to form alanine. Such a transformation could be mediated only through the action of a decarboxylase.

The chromatographic separation of alanine, aspartic acid, and glutamate was supplemented by an electrophoretic separation. Using the electrophoretic technique previously described for the separation of monoamino-monocarboxylic amino acids, complete validation of the chromatographic results was accomplished as shown in Figure 3. A decrease in glutamate intensity and increase in alanine and aspartic acid intensities were noted.

B. Keto-acids

The keto-acid determinations essentially follow expectation if transamination is, in fact, operative in the slime mold.

When tissue extracts were incubated in 1/40 *M* glutamate and 1/10 *M* oxaloacetic acid, Reaction II, the results as shown in Figure 4 were achieved. Alpha-ketoglutarate, one of the end products of the reaction, is seen to increase in intensity with increasing time of incubation of the tissue. The degree of color intensity of the soluble series remained higher than that in the insoluble series. However, oxaloacetate, one of the initial reactants, did not display any reduction in intensity as might have been expected. Since our method did not distinguish between pyruvate and oxaloacetate (see Figure 4), pyruvate may have been generated during the course of the reaction. This would again not be unreasonable in view of the previously stated possibility that oxaloacetate may be decarboxylated to yield pyruvate in the slime mold.

Various concentrations of oxaloacetate were used in addition to the 1/10 *M* concentration. When a lower concentration (1/20 *M*) was used, no spot was evident at the oxaloacetate locus chromatographically. In addition, the enzymatic conversion of this substrate took place rapidly, for alpha-ketoglutarate appeared after only 15 minutes of incubation. Increasing the concentration of oxaloacetate to 1/5 *M* seemed to have an inhibitory effect on the reaction, as indicated by (1) large spots at the oxaloacetic acid locus, (2) extremely small ketoglutarate spots, and (3) no alanine or aspartic acid being formed. On the basis of these test concentrations, 1/10 *M* oxaloacetate was chosen as being the optimal concentration.

Figure 5 shows the results of incubating homogenized tissue with 1/40 *M* glutamic acid and 1/10 *M* pyruvate. Here, as in the case of Reaction II, alpha-ketoglutarate increased in color intensity with increasing lengths of incubation times.

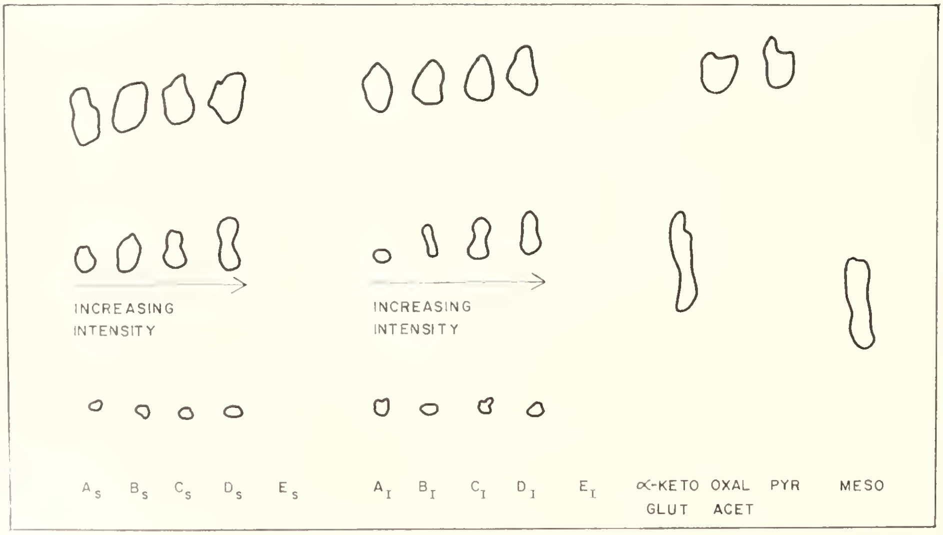

FIGURE 4. Exact reproduction of chromatogram showing keto-acid results of Reaction II. Incubation times same as in Figure 1. α-KETOGLUT = α-ketoglutarate known; OXALACET = oxaloacetic acid known; PYR = pyruvic acid known; MESO = mesoxalic acid known. Mesoxalic acid known was spotted to aid identification of unknown spots. Note loci of unknown "spot B" at points of spot application.

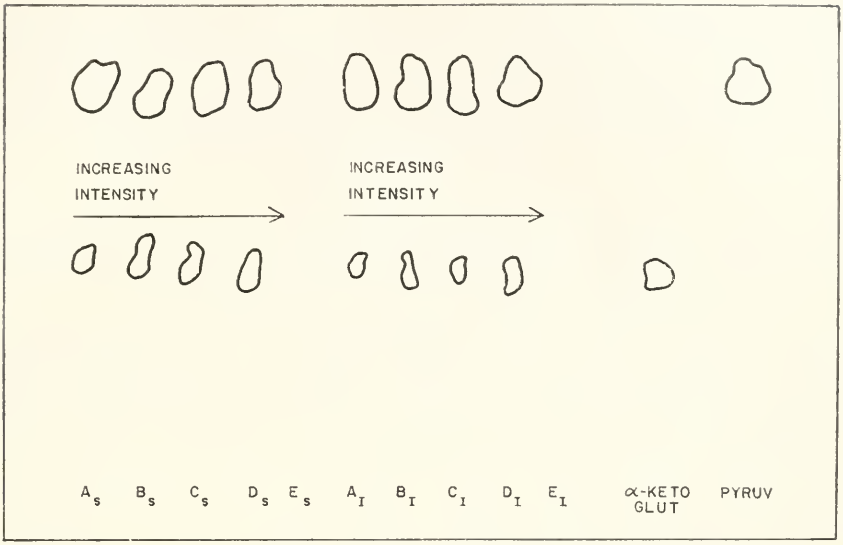

Figure 5. Exact reproduction of chromatogram showing keto-acid results of Reaction I. Incubation times same as in Figure 1. Abbreviations same as in Figure 4.

With pyruvate, as with oxaloacetate at this concentration, no progressive diminution in color intensity occurred with increasing lengths of incubating times. Similar reasoning and possible concentration effects can be applied to this event as were applied to the oxaloacetate results.

Brief mention should be made of two unidentified spots evident only in Reaction II experiments. Their possible importance lies in the fact that they occurred only in the experimental series and were not evident in the controls. The locus of "spot A," when present, was invariably midway between the point of origin (point of application of the test solution on chromatogram) and the alpha-ketoglutarate locus. Although every attempt was made to reproduce exact conditions between each run, "spot A" was not always detected, occurring more times than not. It appeared when using 1/20 *M* and 1/10 *M* concentrations of oxaloacetic acid.

The second unidentified spot, "spot B," was consistently present, its locus being at or slightly above the points of application of the experimental samples on the chromatogram (see Figure 4). The various concentrations of oxaloacetic acid did not affect its appearance. Not only was it present in all test series, but there was also a tendency for it to increase in color intensity with increasing incubation times. Its absence from the controls and from the point of application of a known oxaloacetic acid solution is to be especially noted. The significance, if any, of unidentified "spots A and B" is at present not apparent.

Summary

Progressive chromatography and paper electrophoresis techniques have demonstrated qualitatively the occurrence of two transaminating systems in the slime mold, *Dictyostelium discoideum.* These systems are glutamic-aspartic (or glutamic-

oxaloacetic) transaminase and glutamic-alanine (or glutamic-pyruvic) transaminase. However, in experiments designed to demonstrate the glutamic-aspartic transaminase, alanine was also produced, indicating the presence of an oxaloacetic-pyruvic decarboxylase. The evidence for transaminases confirms the existence of pathways for the conversion of protein to carbohydrate in the slime mold.

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THE OVARY AND ANAL PROCESSES OF "CHARACODON" EISENI, A VIVIPAROUS CYPRINODONT TELEOST FROM MEXICO[1]

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Early classifications of the Mexican fishes of the family Goodeidae, such as those of Jordan and Evermann (1896–1900), Meek (1902, 1904), Regan (1906–1908) and Hubbs (1924, 1926), were based largely on characteristics concerned with the type of jaws, teeth, length of intestine, etc. Actually, many of the species were placed in genera now included in entirely different families, such as the Poeciliidae. Meek recognized the natural relationships of the Goodeidae, using such criteria as (1) viviparity, (2) specialization of the anal fin, and (3) geographic distribution, although he continued to base his classification on the older criteria.

In 1939 Hubbs and Turner revised the taxonomic structure of the goodeids, basing the new classification primarily on characteristics of the ovarian structure and the trophotaeniae, processes extending from the peri-anal region in the embryo and assumed to be used for respiratory and nutritive functions during gestation. The authors concluded that the ovarian and trophotaenial characters indicated the lines of phyletic relationships better than previously used taxonomic schemes. This new classification has been used by workers since 1939. However, De Buen published a key to the family (1942–1943) in which he used the Hubbs-Turner criteria to distinguish genera but reverted to the more usual characteristics to distinguish species. On the other hand, in his recent key to the fishes of Mexico, Alvarez (1950) used the customary taxonomic features but did not refer to the Hubbs-Turner criteria.

Recently there has been some question about the validity and classification of *"Characodon" eiseni* Rutter, synonymized by Hubbs and Turner (1939) with *Characodon variatus* (= *Xenotoca variata*) of previous classifications (Meek, etc.). The need for a careful study of this species was suggested to me by Robert R. Miller of the University of Michigan. It was agreed that I would examine the ovary and trophotaeniae whereas Dr. Miller would reappraise the taxonomic position of the species on the basis of other characters. For various reasons, it has been decided that this portion of the study should be published now, to be followed later by Dr. Miller's taxonomic analysis.

In the process of comparing the ovarian and trophotaenial characteristics of *"Characodon" eiseni* and *Xenotoca variata,* certain discrepancies in structure have become apparent to the writer: (1) there are serious differences in the ovarian and trophotaenial structures of the species described here, *"Characodon" eiseni,* and those of *Xenotoca variata* with which it has been synonymized by Hubbs and Turner

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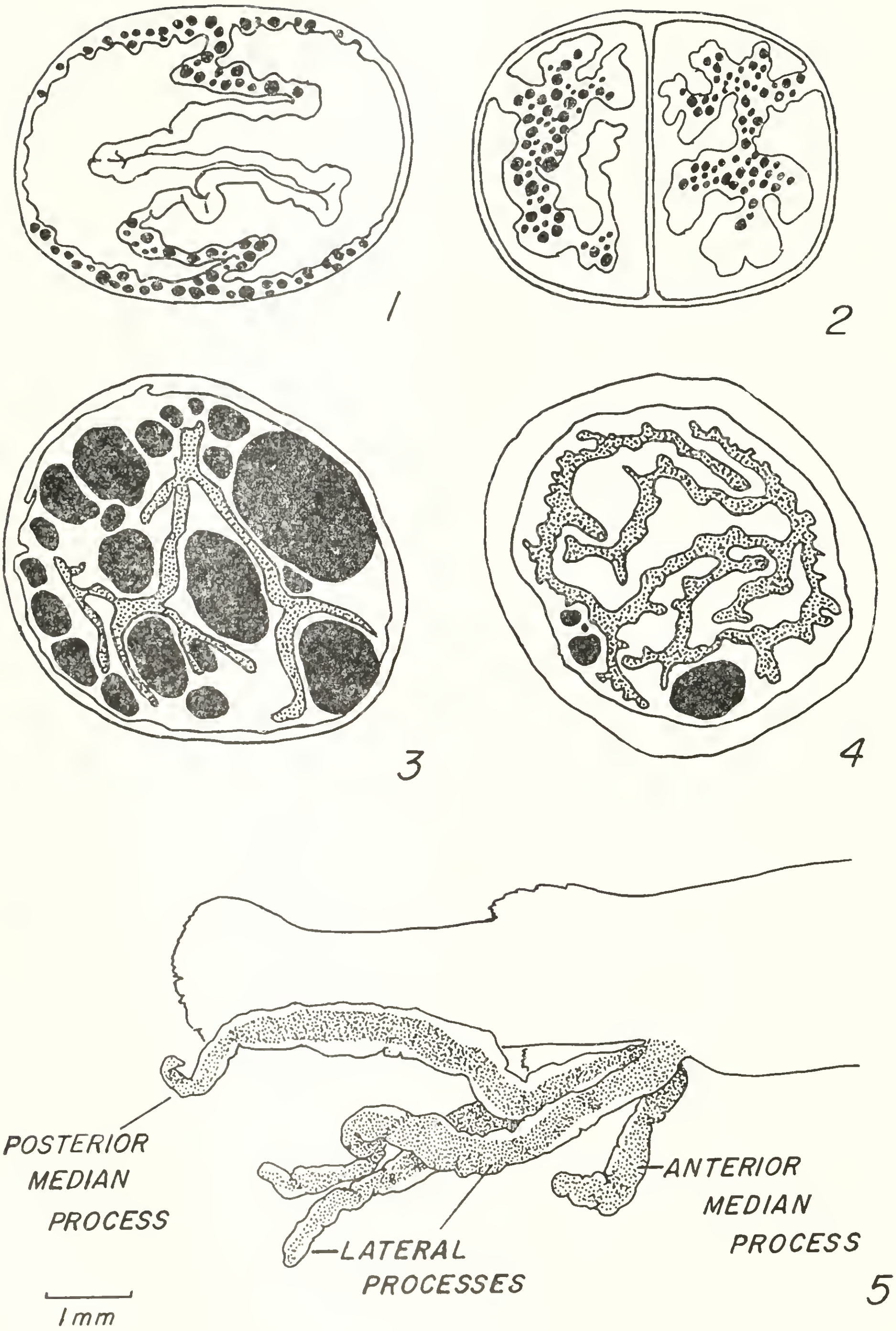
1
2
3
4
POSTERIOR MEDIAN PROCESS
LATERAL PROCESSES
ANTERIOR MEDIAN PROCESS
5
1mm

PLATE I

(1939); (2) regardless of the identity of the species, lack of agreement between the ovarian and trophotaenial structures calls into question the applicability of the Hubbs-Turner criteria in this particular species.

THE GOODEID OVARY AND TROPHOTAENIAE

The ovary of the goodeid fishes is a single, hollow, spindle-shaped structure, continuous posteriorly with the oviduct which in turn opens to the outside at the genital pore immediately behind the anus. The ovary is further divided into two lateral halves by a median vertical septum. The nature of the median septum is very important in the Hubbs-Turner classification scheme. The septum may be single, complex and attached at the mid-dorsal and mid-ventral lines as in *Alloophorus robustus* and *Goodea luitpoldii* (Plate I, Fig. 1) or it may be divided into dorsal and ventral halves as in *Xenoophorus captivus* and *Neoophorus diazi* (Turner, 1933; Hubbs and Turner, 1939). The halves may then be long or short and may be rolled in one lateral direction or the other; other variations occur. A second important characteristic of the ovary is the location of the ovigerous tissue. Eggs may be found in the walls of the ovary (*e.g., Alloophorus robustus* (Plate I, Fig. 1); in some species they may also be found in the septum (*Goodea luitpoldii* and others). In species such as *Neotoca bilineata* (Plate I, Fig. 2), the median septum is thin and bears no eggs; germ cells are restricted to two lobulated folds that protrude into the ovarian lumen from the dorso-lateral walls of the ovary (Turner, 1933; Mendoza, 1940).

In addition to these characteristics, the trophotaeniae were also used by the authors in the classification of the species. These trophotaeniae usually are extensions of the peri-anal lips and may occur in one of two basic forms; they may have the form of a small flower or "rosette" as in *Goodea luitpoldii* (Plate II, Fig. 6), *Neoophorus diazi* and *Allotoca dugesii* (Turner, 1937, Hubbs and Turner, 1939), or they may have the shape of a ribbon, the number of ribbons varying with the different species. For example, *Characodon lateralis* (Turner, 1937) and *Hubbsina turneri* (Mendoza, 1956) have only two posteriorly directed processes (Plate II, Fig. 8); *Neotoca bilineata* (Turner, 1937) and others have three processes in the form of a "trident" extending caudad (Plate II, Fig. 7), but *Zoogoneticus cuitzeoensis* (Plate II, Fig. 11), on the other hand, has 10 to 12 processes (Turner, 1937). Furthermore, the ribbon-shaped processes may be sheathed, in which case the epithelium of the process is separated from the central medulla by a space as in *Neotoca bilineata* and *Skiffia lermae* (Plate III, Figs. 12–13). In non-sheathed

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FIGURES 1–2. Diagrammatic transverse sections of two goodeid ovaries (from Hubbs and Turner, 1939) to show the basic structure of the ovary and the location of the ovigerous tissue. Eggs are shown in black.

FIGURE 1. *Alloophorus robustus.*

FIGURE 2. *Neotoca bilineata.*

FIGURE 3. A section of an immature ovary of *"Characodon" eiseni*, showing a large number of eggs in the anterior region where the median septum is not well formed.

FIGURE 4. A section of a mature post-partum ovary of *"Characodon" eiseni*, showing few eggs in the ovarian wall and a much-folded median septum. Figures 3 and 4 are tracings from photographs; in these figures the ovarian lumen is stippled.

FIGURE 5. The trophotaeniae of *"Characodon" eiseni*. The sheathed nature of the processes shows clearly. The drawing is a tracing of a photograph.

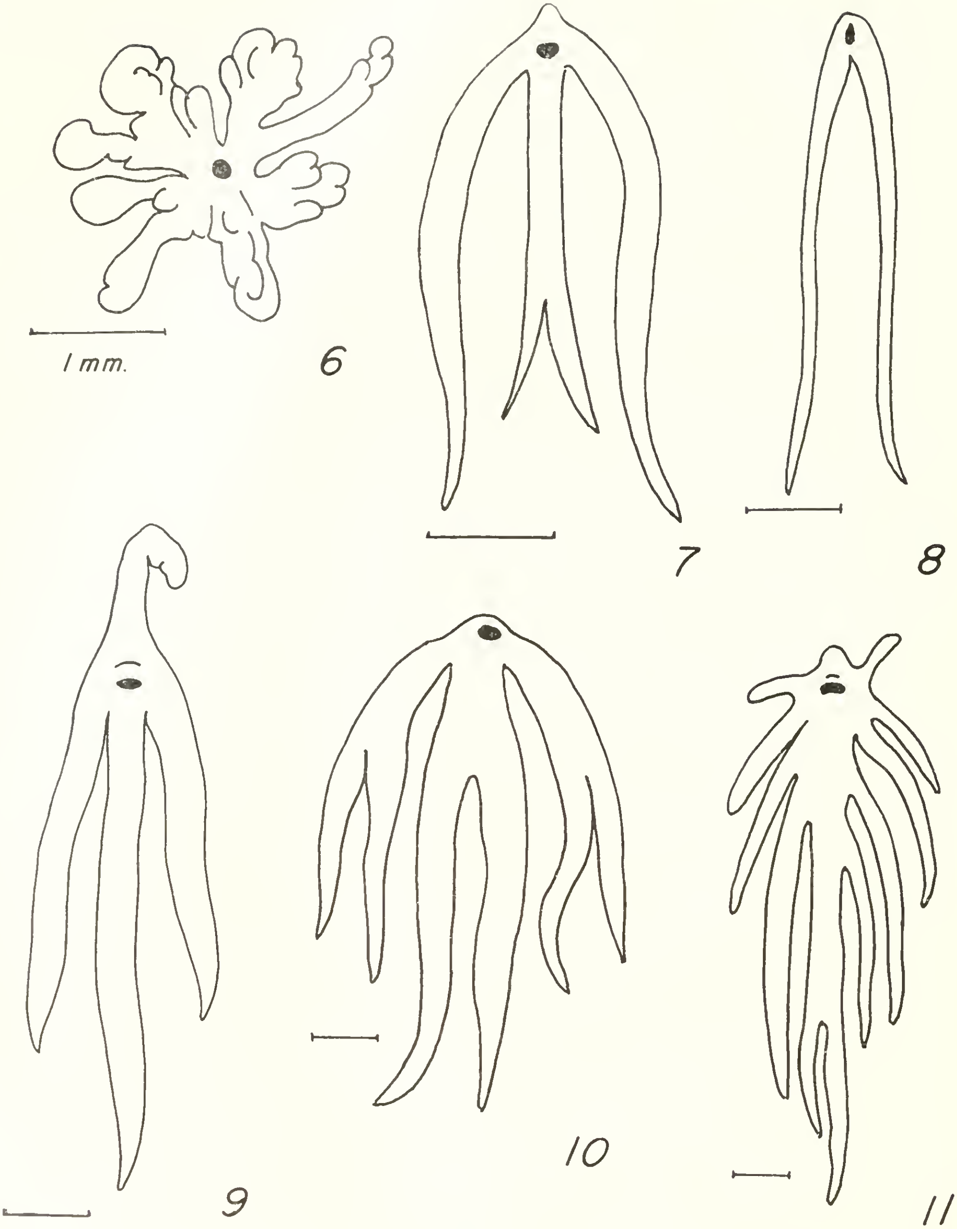

PLATE II

Figures 6–11. Representative types of trophotaeniae from different goodeid species. All figures except 9 are taken from Hubbs and Turner (1939).

Figure 6. *Goodea luitpoldii.*

Figure 7. *Neotoca bilineata.*

Figure 8. *Characodon lateralis.*

processes found in species such as *Alloophorus robustus* (Plate III, Fig. 14), *Zoogoneticus cuitzeoensis,* etc., the epithelium is immediately adjacent to the central core; there is no subepithelial space (Turner, 1937; Hubbs and Turner, 1939).

MATERIALS AND METHODS

All specimens used in this study, living and preserved, were obtained from Robert R. Miller, Curator of Fishes, Museum of Zoology, University of Michigan. The material examined came from the Manantial "El Sacristan" at Tepic, Nayarit, near the type locality for Rutter's species. The writer expresses his gratitude to Dr. Miller for the specimens, for the suggestion that this study be made and for valuable suggestions made during the writing of this manuscript.

The description of the ovary in the present paper is based on a study of approximately 75 gonads. Over 40 ovaries from preserved specimens were studied *in toto*; the others were sectioned, stained by standard techniques and examined microscopically. The nature of the median septum is best analyzed in a whole gonad by removal of the embryos and by examination of the entire organ under a dissecting microscope. Analysis of the septal structure solely from microscopic sections would be very tedious at best and probably very unreliable. For the study of the processes, at least 200 embryos were examined, ranging from neural tube stages to specimens ready for birth (13–14 mm.); observations were made on living, preserved and sectioned specimens. Since the processes undergo serious changes just prior to birth, it is imperative that description of the processes be based on embryos less than maximum size.

OVARY

Gross structure

The ovary is a spindle-shaped organ attached by a strong band of connective tissue to the anterior wall of the body cavity. Two median mesenteries further support the ovary; one is a very short membrane to the pigmented roof of the coelom, the other is a long mesentery to the posterior section of the gut.

In a mature female the resting ovary normally measures 2–3 mm. in diameter; the length of the combined ovary and oviduct varies from 10–30 mm., depending on the size of the female. A gonad with developing embryos varies according to the size of the female and the age and number of the contained young. A representative measurement of an ovary of a 55-mm. female with embryos 11–13 mm. long is 20 × 10 mm. (length by diameter).

The ovary is a typical goodeid ovary; it has a muscular wall, a central lumen and a median septum (Plate I, Figs. 3–4). The gonad is divided approximately into equal halves by a much-folded longitudinal, median septum This membrane is quite variable in its structure for it may be complete, only partially complete or fully divided into dorsal and ventral halves. A complete septum is one that extends the length of the ovary as a single, continuous sheet. However, the septum

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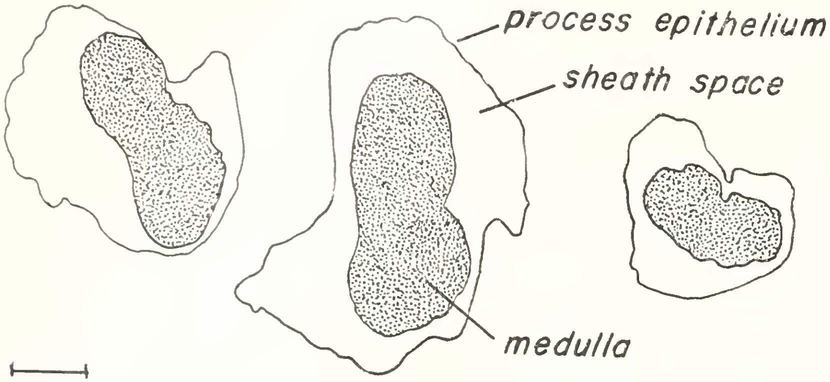
FIGURE 9. *"Characodon" eiseni.* This is a semi-diagrammatic, ventral view of the processes in Figure 5.

FIGURE 10. *Xenotoca variata.*

FIGURE 11. *Zoogoneticus cuitzeoensis.*



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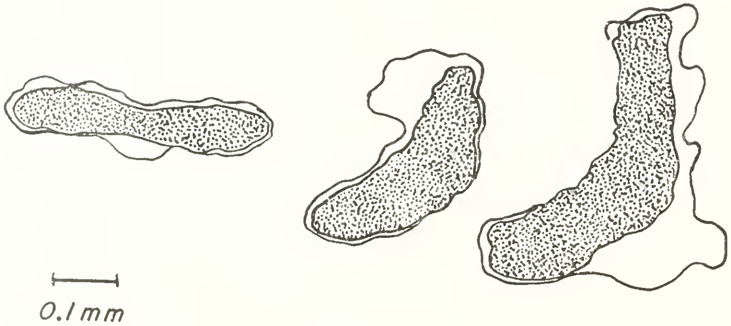

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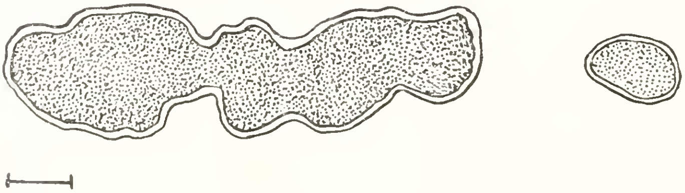

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PLATE III

FIGURES 12–14. Sections of trophotaeniae of three goodeid species. All drawings are tracings from microprojections.

FIGURE 12. *Neotoca bilineata* (11 mm.). Note the delicate epithelium and the generous sheath space around the medulla.

FIGURE 13. *Skiffia lermae* (6 mm.). Sheathed processes similar to those of *Neotoca bilineata*.

FIGURE 14. *Alloophorus robustus* (10 mm.). The process epithelium is thick; the sheath space is absent.

may be complete but perforated by one or more openings of various sizes, usually at the posterior end. If partially complete, the septum is normally intact in the anterior region but is divided into dorsal and ventral halves in the posterior region of the ovary. All possible gradations occur in the degree of completeness of the septum; as little as 25% or as much as 90% of the septum may be intact. If the septum is not complete, it is divided into dorsal and ventral components which may be approximately equal in size or markedly unequal. Among the 43 dissected specimens the following variations in the septum were found:

Table I

Structure of the median septum

Number of ovaries	Condition of median septum
12	Complete; intact the full length of the ovary.
13	Partially complete; some perforations and partial division into dorsal and ventral halves.
14	Divided into two complete and equal halves.
4	Divided into two complete but unequal halves.

Despite variations, the total height of the septum is much greater than the diameter of the ovary, thereby throwing the septum into many folds. Side extensions or branches of this membrane are numerous.

Ovigerous tissue occurs more often in certain locations but it is also quite varied in its distribution. Eggs invariably are found in the anterior half or third of the ovary although they may extend throughout most of the gonad in juvenile specimens. Eggs occur in the ovarian wall and in the septum but more often they are found in the anterior, ventral and lateral walls of the ovary. Eggs that occur in the septum are confined primarily to the ventral edge but they may occur anywhere along the septum (Plate I, Fig. 3).

Histology

Histologically, the mature ovary resembles other goodeid ovaries (Turner, 1937; Hubbs and Turner, 1939; Mendoza, 1940, 1956). In a non-gravid ovary both the septum and the internal walls are extensively folded. The stroma of the gonad is formed of a delicate network of collagenous, mesenchyme-like connective tissue that contains the many eggs and, in mature ovaries, many large blood vessels. A large artery and vein follow a path along the mid-dorsal and mid-ventral lines of the gonad, embedded in the muscular wall. The internal epithelium is squamous or low cuboidal; nuclei are large, rounded and vesicular. The epithelium evidently does not attain the elaborate structure found in *Neotoca bilineata* (Mendoza, 1940). A very extensive capillary plexus lies in a sub-epithelial position in the septum and in the internal ovarian wall; the plexus is very conspicuous in the mature ovary but poorly developed in the immature gonad. Nests of early oogonia occur in the ovarian wall and the septum; eggs attain a maximum size of 250–300 μ. The follicle that surrounds each egg is squamous in smaller eggs but columnar to compound in eggs of maximum size. A thin vascular connective tissue "theca" surrounds each follicle. A spongy or tumescent condition of the ovary occurs only in early stages of development; in advanced stages of gestation the ovarian walls and septa are thin and collapsed. The muscular wall of the mature gonad is very thick and is formed of smooth muscle and connective tissue. In the ovary proper the muscle cells tend to run in a circular manner but there is much random orientation; actual whorls of cells and longitudinally oriented cells occur at random in the muscular layer. A heavy layer of connective tissue borders the muscle layer on the external and internal surfaces; connective tissue fibers also occur in the muscle layer. In the juvenile ovary the muscular wall is very thin. In the region of the oviduct, the smooth muscle cells in the wall are arranged in two orderly layers; one is longitudinal, narrow and external in position, the other is circular, wide and internal.

TROPHOTAENIAE

There are four basic processes; one is median in position and anterior to the anus; the other three extend posteriorly; two are lateral and one is median and posterior to the anus (Plate I, Fig. 5). Any one process may be modified, degenerate or completely missing. Any process may be secondarily split, the point of bifurcation occurring at a proximal or distal position along the process. Splitting is more likely to take place in one rather than in two or more processes at one time and, although splitting may be found in any process, the total number of ribbons seldom exceeds six. Sometimes two or even three of the processes arise from one common base.

The anterior process is invariably short; the posterior median process tends to be the longest but the lateral processes approach or may even exceed it in length. At the point of maximum development, one or more of the processes extend to the caudal fin and often extend beyond the tip. This size relationship is true for embryos at all lengths, 6 mm. or 13 mm. The following examples are illustrative of processes in embryos 12–13 mm. long. The maximum length recorded for any process was 7.5 mm. in a 13-mm. embryo ready for birth. The maximum length is normally retained until time for birth although embryos frequently begin to resorb the processes even before birth; some specimens just prior to birth have been observed with processes that extend only to the anal fin. Processes normally measure 0.3–0.4 mm. in typical maximum width although some of 1.0 mm. have been observed in exceptional cases. At optimal development, processes appear turgid, smooth, translucent; as birth approaches, they become compact, less translucent and have a "furry" appearance.

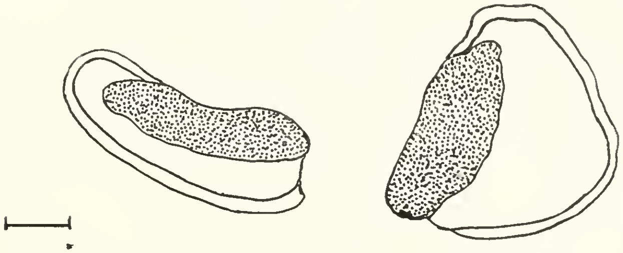
TABLE II

Length of anal processes in millimeters

Specimen	Anterior median	Right lateral	Left lateral	Posterior median
1	1.5	4.5	5.0	4.5
2	1.5	5.5, 6.5	5.5	6.0, 5.0
3	1.5	6.5	6.0	6.0, 7.0
4	2.5	5.5	6.0	5.0
5	1.0, 1.0*	5.0	4.0, 4.0	5.0

* Double figures indicate split processes.

Processes are unquestionably sheathed; a central medulla is separated by a sheath space from the epithelial covering (Plate I, Fig. 5). The sheath is normal for specimens up to stages approaching birth; at this time, the characteristic may be lost. The sheath characteristic may be visible even in the proximal peduncle that forms the base of the processes. The sheath space may be extensive and continuous or broken up into smaller vesicles (Plate IV, Figs. 16–17). The medulla or core



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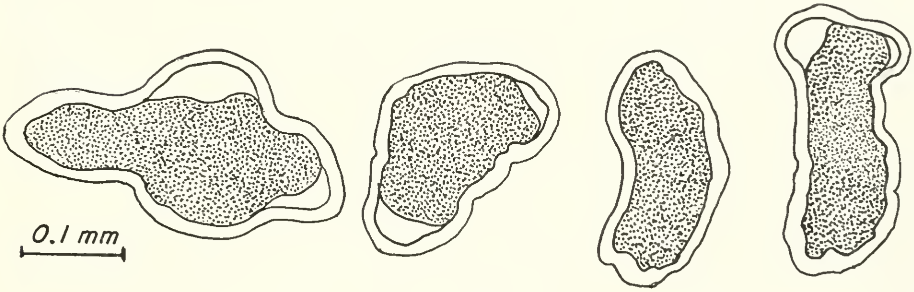

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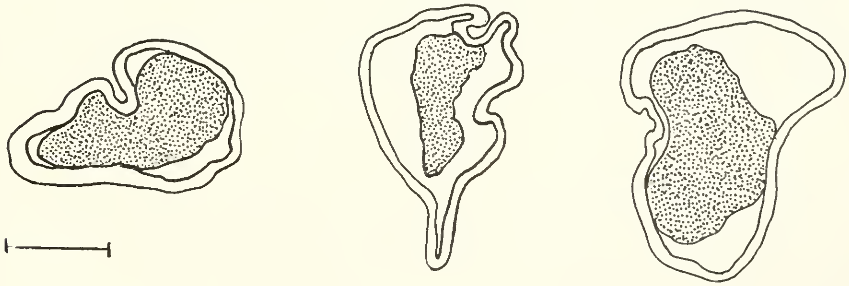

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PLATE IV

Figure 15. Trophotaeniae of *Goodea luitpoldii* (8 mm.). This "rosette" type process has a large sheath space in small embryos but the space is absent in older embryos.

Figures 16–17. Trophotaeniae from two 8-mm. specimens of *"Characodon" eiseni.* The epithelium shown is thick; the sheath space is variable in appearance; it is generous, restricted or absent in some regions. All figures are tracings from microprojections.

normally measures 0.16–0.24 mm. in maximum width although some measurements of 0.35 mm. have been noted. The medulla is normally attached to the dorsal epithelium although it may attach to any area of the epithelium. On occasions, the medulla may even protrude beyond the surface of the process, carrying the process epithelium out with it.

Histology

Two types of cells form the epithelium of the processes; one is an extension of the gut epithelium, the other is a continuation of the epidermis. Because of the origin of the processes, the former is found on the ventral surface of the processes, the latter on the dorsal surface. The cells derived from the gut epithelium are cuboidal to low columnar and are normally 8–10 μ high; the nucleus is primarily spherical, basal in position, vesicular and 3.5–5.0 μ in size. The cells show a conspicuous "brush border" that, in the light of modern microscopy, is probably a surface covered with microvilli. The position of the nucleus and the stratification of the heterogeneous cytoplasm are evidence of a physiologically active cell. The basement membrane of these cells is extremely delicate. The fact that mitotic figures are seldom seen indicates that the processes probably grow at the base. The epithelium derived from the epidermis is very thin, composed of flattened cells normally arranged in an irregular double layer and often vacuolated. The transition between the two types of cells is abrupt. The large cuboidal cells normally form 75% or more of the epithelial surface. The most typical appearance of the epithelium occurs in embryos 10 mm. or less in length; as time approaches for birth, the epithelium and, indeed, the entire process undergo marked changes.

The medulla is formed of a mass of loose, spongy, connective tissue. Fibrocyte nuclei are approximately 10 μ in length, oval, pale, finely granular and homogeneous in appearance. Approaching birth, many phagocytes appear in the tissues. As is true for other goodeids, the blood supply to the processes is very rich, forming an extensive capillary plexus on the medullary surface. The vascular character is a property of the medulla, not of the epithelium. Occasionally, capillaries or large vessels protrude beyond the surface of the entire process in the region of the "epidermal" epithelium. The medullary connective tissue is continuous with the submucosa of the gut and the sub-epidermal connective tissue of the body surface.

DISCUSSION

The present description of the ovary and the trophotaeniae of "*Characodon*" *eiseni* differs from that given for *Xenotoca* (*variata*) by Hubbs and Turner (1939) in some respects; there are two serious differences and other minor ones. In their study, the median septum of *Xenotoca* is described as "entire, attached dorsally and ventrally, much folded" (Hubbs and Turner, 1939, Table II). This property places *Xenotoca* in the second phyletic line, along with *Alloophorus* and *Chapalichthys*. However, in the present study only 12 of the 43 ovaries were found to follow this description. An additional 13 ovaries had a septum essentially complete but with minor or more serious variations, whereas 18 ovaries had a septum divided distinctly into dorsal and ventral halves. In this species, therefore, the median septum is inconsistent or variable in form and thus is an unreliable criterion for use in classification. Using this criterion, *"Characodon" eiseni* may well be classified in three phyletic lines, numbers 2, 6 or 7 (Hubbs and Turner, 1939, Table II).

No serious discrepancies were found in the description of the location of the ovigerous tissue although it appears to the writer that, except for the extreme anterior end, eggs seldom occur in the median septum; they occur mostly in the walls of the ovary and especially at the anterior end. This again differs somewhat from the revision of Hubbs and Turner where *Xenotoca* is likened to *Alloophorus* and the latter is described as having ovarian "walls . . . almost devoid of ovigerous tissue" (p. 13).

Hubbs and Turner describe the trophotaeniae of *Xenotoca* as 6 to 8 in number, very long and unsheathed (Plate II, Fig. 10). The processes are said to "arise by dichotomous branching from three backwardly projecting trunks, one median and two lateral" (p. 25). Having examined more than 200 specimens, the writer concludes that there are four basic processes in *"Characodon" eiseni,* the one posterior median process and two lateral processes as described by Hubbs and Turner for *Xenotoca* but with an additional median process anterior to the anus. The writer agrees with Turner that there is much secondary splitting (Turner, 1937). Furthermore, although the writer agrees that there may be as many as 6 to 8 processes, this is a number seldom attained; 4 to 6 processes is a much more representative number. The slight disagreement in number is a minor matter but it is important that a fourth antero-median process be recognized as part of the basic set of processes.

Another serious difference arises in the matter of the presence or absence of the sheath around the process. *Xenotoca* is described as having unsheathed trophotaeniae (Turner, 1937; Hubbs and Turner, 1939), thereby placing it in a category with *Alloophorus robustus, Chapalichthys encaustus,* and *Zoogoneticus cuitzeoensis.* The size of the embryos, the stage of development and the number of specimens examined may well affect the conclusions drawn. In embryos of *"Characodon" eiseni* approaching birth, the processes do tend to show an absence of a sheath but in younger stages there is no question of the presence of a sheath, although even this is variable in degree of formation. Following Hubbs and Turner, the presence of the sheath in the processes should place this species with genera such as *Skiffia, Ollentodon* and *Neotoca,* in the subfamily Girardinichthyinae rather than in the Goodeinae. The three genera listed are the only other ones in which the processes are stated to be sheathed. While this paper is not intended to include an evolutionary analysis of the species, it seems to the writer that the arrangement of processes in *"Characodon" eiseni* could easily have arisen from the "trident" arrangement present in species such as *Neotocoa bilineata,* simply by the addition of a short median process anterior to the anus. Finally, a minor difference arises in regard to the nature of the process epithelium. The writer is not in agreement that the ". . . epithelium . . . is everywhere simple and of irregular height" (Hubbs and Turner, 1939, p. 25). In the younger embryos the epithelium has a dual structure, depending on whether it is continuous with the epidermis or the gut epithelium. The double nature is very clear; each of the two types tends to be quite regular in its own structure. The irregularity referred to may be true in stages just before birth when the entire process undergoes marked changes, preceding its resorption at about the time of birth. At this time the epithelium does become most irregular and is even sloughed off. The writer appreciates the fact that this is a minor matter, a detail readily noticed in a descriptive, histological study but likely to be missed in a paper of broader scope and concerned primarily with overall taxonomic matters.

In conclusion, the writer points out the overall taxonomic impasse in which these newer facts place *"Characodon" eiseni.* First, the description of the median septum of the ovary is not in agreement with that given for *Xenotoca variata* by Hubbs and Turner (1939). Second, because of the variability of the structure of the median septum, *"Characodon" eiseni* can be placed in different phyletic lines within the family as determined by the nature of the median septum. Hence this criterion is unreliable for the classification of this species. Third, the sheathed processes found in *"Characodon" eiseni* are totally different from the solid, non-sheathed processes described for *Xenotoca variata.* Fourth, the presence of sheathed processes in this species is inconsistent with the type of median septum described here for *"Characodon" eiseni* or that of *Xenotoca variata.* In the classification scheme devised by Hubbs and Turner, species that have sheathed processes have an ovary with a thin, delicate, median septum and ovigerous tissue confined to two dorso-lateral folds (*e.g., Neotoca bilineata*). Such an ovary has but little in common with that described for either *"Characodon" eiseni* or *Xenotoca variata.* Thus two major criteria (ovarian structure and type of process) are at odds with each other and one criterion (ovarian structure) fails to discriminate between two or more phyletic lines.

The facts brought out in this paper raise the serious question whether taxonomic criteria based on ovarian and trophotaenial structures can be used successfully in the case of this species. At the same time, this paper does not propose to extend this conclusion to the entire goodeid family since this study is limited only to one species, *"Characodon" eiseni.* It may well be that a restudy should be made of the degree of variation in the structure of the septum in some or most species of the family, particularly those species in which the septum is ovigerous. It is not likely that a median septum of the *Neotoca bilineata* type (thin and non-ovigerous) will vary much. The criteria set up by Hubbs and Turner in 1939 no doubt will still prove to be valuable, even though there may be exceptional forms such as this species in which the criteria are not absolutely discriminatory. Lastly, a taxonomic analysis of this species using other conventional criteria should help to clarify the taxonomic relationships of *"Characodon" eiseni* and *Xenotoca variata.*

SUMMARY

The ovary and trophotaeniae of *"Characodon" eiseni* Rutter are described. The median septum of the ovary is variable in structure; the septum may be a single, continuous sheet or it may be divided into dorsal and ventral halves. Ovigerous tissue is confined primarily to the anterior region of the ovary but mostly to the ovarian wall. There are four trophotaeniae (processes), two lateral and two median, one anterior and one posterior to the anus. The processes are further described as sheathed. The above facts are not in agreement with previously published descriptions of *Xenotoca variata* with which this species has been synonymized. The above facts are further contradictory with each other in assigning

"Characodon" eiseni to a particular evolutionary line within the family Goodeidae. The paper shows that the goodeid taxonomic criteria based on ovarian and trophotaenial structure are not discriminatory when applied to *"Characodon" eiseni.*

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SYMBIOSIS OF HYDRA AND ALGAE.
II. EFFECTS OF LIMITED FOOD AND STARVATION ON GROWTH OF SYMBIOTIC AND APOSYMBIOTIC HYDRA

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Very few measurements have been made on the effect of symbiotic algae on growth of their various invertebrate hosts. Recently Karakashian (1963) demonstrated that the algae symbiotic with *Paramecium bursaria* exert a strong influence on the growth of the host. These studies were carried out with symbiotic and aposymbiotic individuals of known genetic and nutritional history cultured in a defined medium. Culture techniques (Loomis, 1954; Muscatine and Lenhoff, 1965) now permit a similar approach using green hydra, thus affording insight into an association of algae and a metazoan. Previous studies on the role of algae in green hydra (Goetsch, 1924; Haffner, 1925) were carried out in undefined media, and lacked the quantitative precision necessary for critical evaluation.

The present study describes experiments on the growth, survival, and protein turnover of hydra with and without algae as a function of exogenous food supply. Possible mechanisms of interaction between algae and host are discussed. A preliminary note on some of this work has appeared elsewhere (Muscatine, 1961).

MATERIALS AND METHODS

All experiments were carried out with *Chlorohydra viridissima,* Carolina strain 1960.The culture medium, and methods for maintaining animals in the laboratory, sampling individuals for experiments, obtaining algae-free controls and conducting growth experiments are described in a previous paper (Muscatine and Lenhoff, 1965).

"Pale green" hydra containing known amounts of algae intermediate between green and albino (= algae-free) were obtained in the following manner. Green hydra were placed in culture solution containing 0.068 *M* glycerine, which causes the gradual elimination of algae (Whitney, 1907, 1908). At daily intervals for eight days, groups of ten "uniform" (*cf.* Lenhoff and Bovaird, 1961) hydra were removed, rinsed in clean culture solution, and exposed to $C^{14}O_2$ for exactly 24 hours, using a procedure described by Muscatine and Lenhoff (1963). These labeled animals were then rinsed in several changes of clean culture solution, and placed on a Millipore filter (HA-47) in a drop of deionized water. When relaxed, the animals were flattened on the filter by application of suction (*cf.* Lenhoff, 1959).

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The filter was dried, glued to an aluminum planchet and assayed for radioactivity. The level of radioactivity of untreated green hydra controls was considered to represent the net photosynthetic activity of the normal algal flora. Glycerine-treated animals having fewer algae had proportionally less radioactivity. Albinos served as controls for animal fixation of $C^{14}O_2$. Figure 1 shows the radioactivity of each group plotted against time grown in glycerinated culture solution. Hydra sampled after four and six days of glycerine treatment were judged to contain, from this reference curve, approximately 10–20% and 4–6%, respectively, of their usual normal complement of algae. These animals were washed with several changes of clean culture solution one hour before experiments.

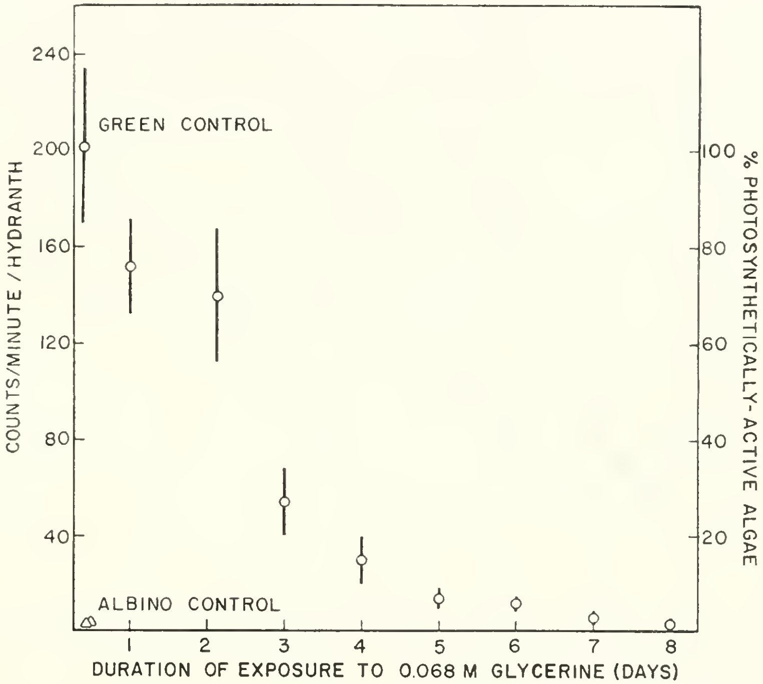

FIGURE 1. Radioactivity accumulated by green hydra exposed first to 0.068 *M* glycerine for periods up to 8 days, and then to $C^{14}O_2$ for 24 hours. Vertical bars denote twice the standard deviation of the mean number of counts per minute. Non-glycerine-treated green controls, in 18 trials, gave 203.2 ±32.0 counts per minute per hydranth. Albinos gave less than two counts per minute above background.

To graft the heads (hypostome and tentacles) of green hydra onto the bodies (gastric region and below) of albinos, one-day starved stock hydra were bisected transversely. Appropriate pieces were threaded on a hair and held together by gentle pressure with watchmaker's forceps. Adhesion began within a minute or two and grafts were available after 15–30 minutes. The approximate algal content of green heads was estimated by first exposing whole intact "uniform" green hydra to $C^{14}O_2$ in a standard manner (Muscatine and Lenhoff, 1963), and then cutting each animal in two just below the hypostome and tentacles. Each head and body was then dried separately on a planchet and assayed for radioactivity. In five replicates, green heads were found to contain 30.5 ± 2.3% of the normal complement of photosynthetically-active algae in an entire animal.

S^{35}-labeled mouse liver (specific activity 1000–2500 counts per minute per microgram protein nitrogen) was prepared, administered to hydra and fractionated as described by Lenhoff (1961). Radioactive material was assayed, with correction for background, with an end window gas flow counter (Nuclear-Chicago C111-B).

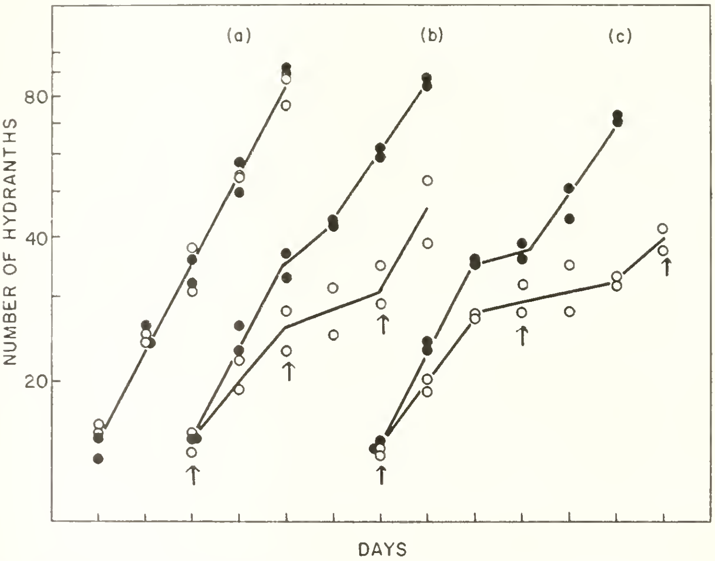

FIGURE 2. Semi-logarithmic plot of growth of green (closed circles) and albino (open circles) *C. viridissima* (a) fed daily, (b) fed every second day, and (c) fed every third day. Arrows indicate time of feeding.

RESULTS

1. *The effect of amount of food on growth of green and albino C. viridissima*

In a previous paper (Muscatine and Lenhoff, 1965) we reported that green and albino *C. viridissima* grew at nearly identical logarithmic rates when fed daily on excess *Artemia* nauplii. This is illustrated in Figure 2, curve a. A doubling time of about 1.5 days is the maximum growth rate (k_{max}) for this species under these conditions (Muscatine and Lenhoff, 1965). Growth of albinos at k_{max} indicates that algae are not essential for logarithmic growth as long as there is ample exogenous food. However, when food was limited, growth rates of green hydra always exceeded those of algae-free individuals, as shown in curves b and c. Curve b shows that the growth rate of green hydra fed every second day deviated only slightly from the rate of animals fed daily. Growth of albinos, on the other hand, lagged after the second feeding, and increased only after a third feeding. Green hydra produced nearly twice the number of buds produced by albinos. When the diet of excess *Artemia* nauplii was further limited to a feeding every third day (curve c), growth of green hydra dropped off sharply during the first two-day interval without food, but resumed a nearly normal rate immediately after the next feeding. Growth of albinos also dropped off after two days without food but continued to lag through the second feeding without resuming a normal rate. Again, green hydra produced almost twice the number of buds produced by albinos.

Since hydra are normally given excess *Artemia* larvae at a feeding, there was the possibility that in experiments with limited feeding, green hydra had simply taken in more food. This was tested by feeding green and albino hydra daily with single *Artemia* nauplii. This regime both controlled and limited the food intake. Freshly hatched larvae were fed to individual green and albino hydra with a tapered pipette allowing the larvae to leave singly. Since the number of hydranths in each culture changed as the experiment progressed, a second shrimp was given to some individuals in order that cultures would receive the same number of shrimp. In this case the additional larvae were fed to maturing buds. Table I shows that under these conditions the average growth rate of green hydra (0.29) still approached that of well-fed individuals, while the average for albinos (0.19) was significantly lower ($p < 0.05$).

TABLE I

Growth of replicate cultures of green (G) and albino (A) C. viridissima fed daily but only on single Artemia nauplii. Numbers in parentheses indicate total number of shrimp given to each culture

		No. of hydranths on day													
Exp.		1		2		3		4		5		6		7	k
			(5)		(9)		(15)		(19)		(23)		(30)		
1	G	10		17		22		30		41		60		71	0.277
	G	10		18		25		31		41		57		71	0.277
	A	10		20		23		25		30		35		36	0.121
	A	10		17		19		22		27		37		39	0.187
			(2)		(3)		(3)		(4)		(5)		(8)		
2	G	4		7		8		10		13		21		—	0.346
	G	4		5		8		9		11		17		—	0.277
	G	4		6		8		9		11		17		—	0.277
	A	4		5		6		6		9		11		—	0.198
	A	4		6		6		7		7		12		—	0.210
	A	4		5		6		7		10		12		—	0.231

Some experiments were carried out to determine if a greater capacity for gastrodermal phagocytosis might have accounted for the increased growth of green hydra on a limited food supply. Twelve green and 12 albino hydra were each fed a small piece of S^{35}-labeled mouse liver along with excess *Artemia* nauplii. Fractionation by differential solubilities showed that 80% of the isotope was bound in the alcohol, trichloroacetic acid-insoluble liver fraction, *i.e.,* the residual protein fraction, and was thus favorable for tracing the course of food protein from the gut lumen into phogacytic digestive cells. At hourly intervals during the six hours following ingestion, duplicate pairs of green and albino hydra were bisected longitudinally, and the gut contents (ingested but not phagocytized) were washed out with culture solution onto a planchet. The radioactivity of this material was then measured and compared to that remaining in the hydra tissues (phagocytized). The curve in Figure 3 represents the rate of phagocytosis of sulfur-labeled tissue. Phagocytosis proceeded relatively slowly over the first two hours, more rapidly during the next two to three hours, and then more slowly after five to six hours as the phagocytic capacity of gastrodermis reached a maximum. Both green and albino hydra phagocytized 85–95% of the labeled tissue and at similar

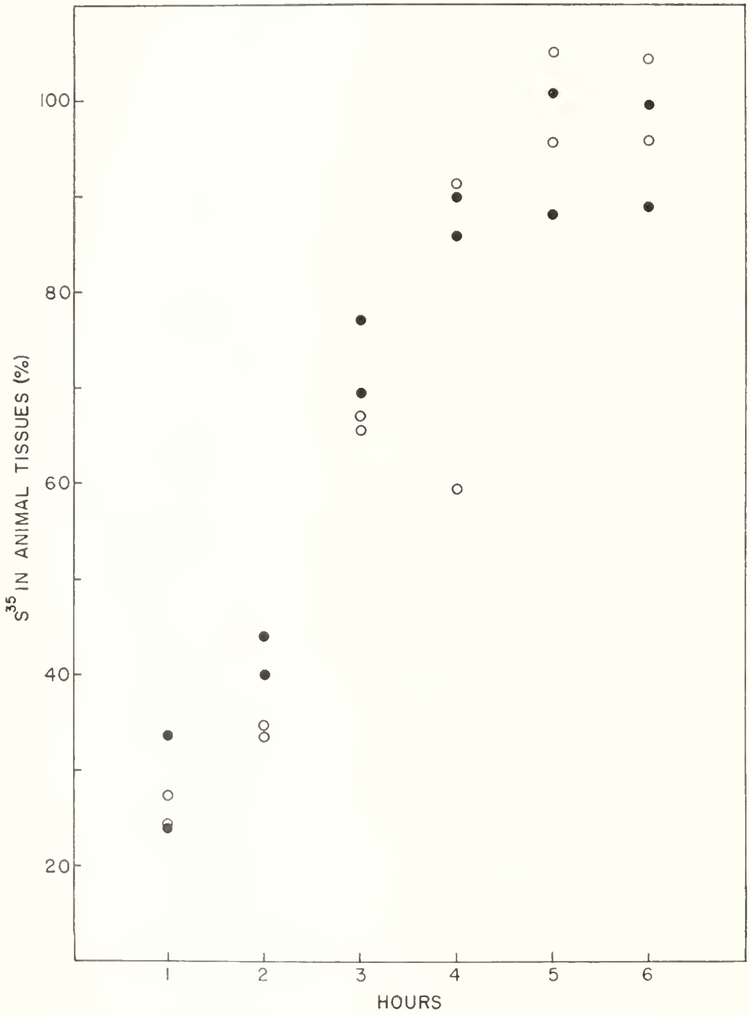

FIGURE 3. Rate of phagocytosis of S^{35}-labeled mouse liver by replicate cultures of green (closed circles) and albino (open circles) *C. viridissima.*

rates, indicating that the absence of algae did not impair the phagocytic capacity of albino *C. viridissima*. Thus, the difference in growth of green and albino on a limited food supply was not simply the result of a quantitative difference in food intake. This conclusion is further borne out by starvation experiments.

2. *The effect of starvation on survival of green and albino C. viridissima*

Goetsch (1924) described an experiment in which green and albino hydra were placed in the same aquarium with little food and the albinos gradually died out. He concluded that albinos live only when well-supplied with food. These observations were confirmed by randomly placing 10 green and 10 albino *C. viridissima* in a 10-gallon aquarium containing aged tap water, several *Gambusia* sp., common aquatic plants, and a sparse population of an unidentified ostracod. This laboratory "ecosystem" was observed daily but otherwise unattended. After three weeks the number of green hydra had at least trebled while no albinos could be found.

Table II

Results of 8 replicate experiments showing the mean (± standard deviation of the mean) number of green, pale green and albino C. viridissima surviving starvation, and the range of survival times

Group	% algae	No. of hydranths on day								Range of survival (days)
		0	2	4	6	8	10	12	14	
Green	100	10	20.5 ±1.5	24.7 ±1.5	28.5 ±1.9	29.5 ±4.3	29.0 ±2.4	32.5 ±0.7	31.0 ±0.0	28–30
Pale green	10–20	10	20.7 ±1.7	24.2 ±3.1	23.7 ±3.4	23.4 ±3.7	23.0 ±3.6	21.0 ±4.9	22.2 ±3.2	24–26
Pale green	4–6	10	21.0 ±1.0	25.5 ±3.5	24.5 ±3.5	24.5 ±3.5	21.0 ±6.0	13.0 ±4.0	8.0 ±6.0	17–20
Albino	0	10	18.2 ±3.5	20.6 ±3.7	18.7 ±4.3	12.5 ±4.2	5.6 ±3.2	1.7 —	0.6 —	10–12

To obtain quantitative data on starvation, five green hydra were placed in 30 ml. of culture solution in a Petri dish (100 mm. × 15 mm.). Five albinos were similarly treated. Also starved in the same manner were two different groups of five "pale green" *C. viridissima*, one containing 4–6% and the other 10–20% of the normal algal flora. The animals were illuminated but not fed, and the culture medium was changed once daily. The number of hydranths in each vessel was recorded daily. The results are shown in Table II. During starvation green hydra produced buds for 12 days and survived for nearly four weeks, gradually becoming smaller during this time, and finally disintegrating. "Pale green" individuals did not appear to change, judging from their relative shades of green, until after about 10 days of starvation when the 5% "pale green" group seemed noticeably whiter. Albinos produced buds for about 6 days and most survived for only 10–12 days. One or two individuals, in subsequent starvation experiments, survived for as long as 17 days. Unlike green and pale green hydra, most of the albinos disintegrated soon after they discontinued budding. This unusually premature event was characterized by crumbling of tentacles and body tube until all that remained of each albino was an amorphous accumulation of whitish debris. It was frequently difficult to decide when an albino was "dead." This was arbitrarily taken as the time at which the crumbling of tentacles was first noticed. Goetsch also observed the disintegration of starving albinos in contrast to the gradual diminution of similarly treated green hydra.

From the data on starvation in Table II, it was possible to estimate the degree to which the number of algae influenced survival. In Figure 4 the percentage of algae contained initially by the hydra in each group is plotted against (1) the average number of hydranths present at 12 days starvation, and (2) the range of maximum survival times. In the first curve, 12 days was chosen because it represents the time at which no albinos remain, although data from 8–14 days give curves of essentially the same character. The shape of the resulting curves is interpreted to mean that survival ability of a starved *C. viridissima* is not appreciably impaired until the level of its photosynthetically-active plant material drops below 15–20% of its normal value. Thus, only about 5–10% of the normal algal flora appears necessary for half-maximum survival ability of the starving host.

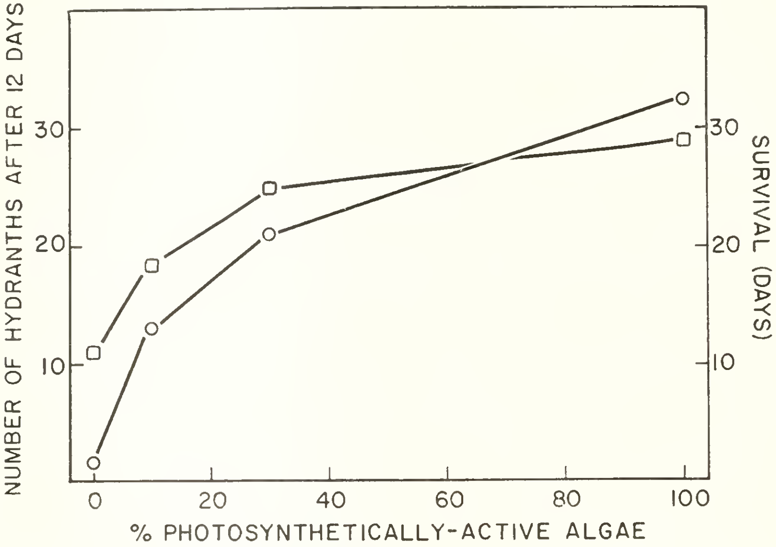

FIGURE 4. Survival of starved *C. viridissima* as a function of the number of algae contained. Using data from Table III, the per cent of photosynthetically-active plant material is plotted against the number of hydranths present after 12 days' starvation (open circles) and mean survival (open squares).

3. *The effect of starvation on turnover of S^{35}-labeled food*

To compare the turnover of protein by starving green and albino *C. viridissima*, we measured the rate at which radioactivity was released into the medium by hydra which had previously ingested S^{35}-labeled mouse liver. Duplicate groups of 10 green, 20 albino, and 10 "pale green" (15% algae) hydra were fed sulfur-labeled liver and allowed to regurgitate the uneaten portion 6 hours later. Immediately after regurgitation 10 of the labeled albinos were decapitated and unlabeled green heads of known algal content were grafted onto the labeled albino bodies as described under Methods. Each group of hydra (green, "pale green," albino, and graft) was placed in 2 ml. of culture solution in depressions of plastic temperature control blocks (Coral Research and Development, Miami, Fla.) maintained at 22.5 ±0.25° C. At 24-hour intervals for five days, the culture fluid was removed from each group and the animals and vessels were rinsed with 0.5 ml. of culture solution per group. The solution and rinsings were combined, dried on planchets and assayed for radioactivity. A fresh 2-ml. portion of culture solution was added to the hydra. After five days the animals were removed and assayed for radioactivity. The sum of the radioactivity of fluid samples is the total S^{35} present at the beginning of the experiment. Material released is expressed as a per cent of this total. In five experiments (Table III) the loss of S^{35} by the groups of hydra always bore the same relationship, although considerable variation was encountered from one experiment to another. Figure 5 shows the rates of loss in one experiment. Invariably albinos lost material to the medium faster than any other group. Pale green and grafted hydra which contained, respectively, 15% and 30% of the normal complement of algae lost material at a lower rate. Green hydra lost material at about half the rate of albinos and retained labeled material about twice as long. The relationship between rate of loss of labeled material by a group of hydra and its algal content is similar to that illustrated by the curves in Figure 4, where relatively few algae have nearly the same effect on the host as does a full complement of algae. The ability of the algae in grafted individuals to modify the rate of loss of material from the labeled albino body, despite the localization of algae in the unlabeled head, exemplifies a "replacement therapy" type of experiment, and suggests that the algae in this case might act by releasing something which diffuses through a distance.

TABLE III

Per cent of S^{35} lost by groups of green, albino, pale green (10–20% algae) and grafted (30.5% algae) C. viridissima during 5 days' starvation

Expt.		Green	Albino	Pale	Graft
1	a	10.8	22.8	—	—
	b	14.2	23.5	—	—
2	a	19.9	40.5	—	—
	b	23.2	33.2	—	—
3	a	19.1	89.5	29.6	28.2
	b	29.9	53.5	24.4	31.0
4	a	12.0	24.6	—	—
	b	14.0	34.8	—	—
	c	11.1	35.4	—	—
5	a	35.2	68.5	57.3	48.1
	b	40.6	82.1	46.3	61.1

That less material appeared in the medium of green and pale green hydra than in the medium of albinos implied that the algae either (1) directly affected the catabolic activities of the host cells, or (2) accumulated the labeled material after it was released by the animal cells. This was investigated in preliminary experiments in which sulfur-labeled hydra were starved for five days and then homogenized by ultrasonic vibration. By gentle centrifugation most of the algae were separated from the bulk of the animal tissues, but mutual contamination could not be avoided. In two trials the resulting algal pellet contained 3.8% and 6.8% of the total radioactivity in the entire homogenate, tentatively indicating that the algae did not accumulate the isotope but depressed the rate of protein catabolism of the host.

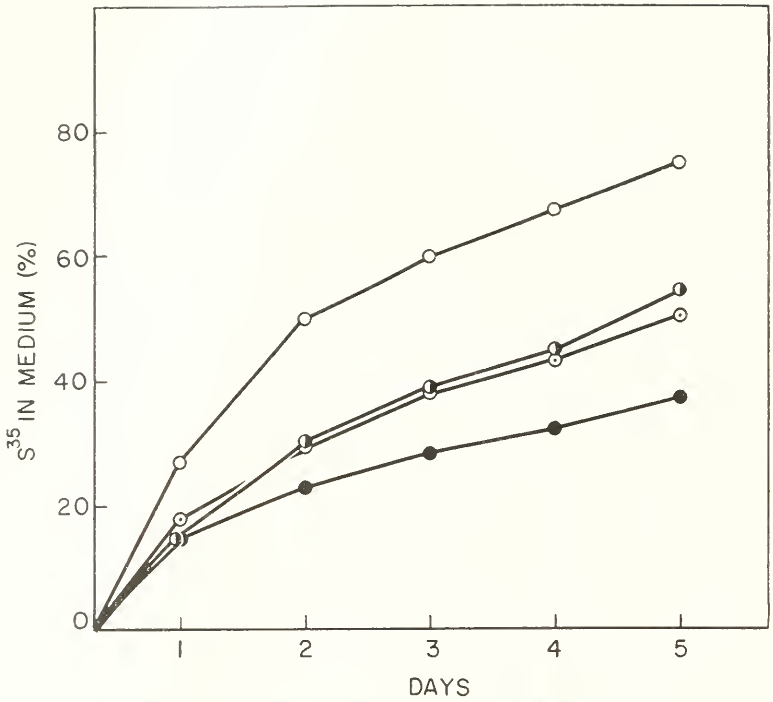

FIGURE 5. Rate of loss of S^{35}-labeled material by green (●), grafted (◑), pale green (⊙), and albino (○) *C. viridissima* containing, respectively, 100%, 30%, 20% and 0% photosynthetically-active plant material.

DISCUSSION

The results of this study lead to the conclusion that symbiotic algae favorably influence the growth, reproduction, survival, and protein turn-over of *C. viridissima*. The growth rate of green *C. viridissima* on a limited food supply was consistently greater than that of aposymbiotic controls (Fig. 2; Table I). This difference was not the result of a proportionally larger food intake by green hydra (Fig. 3), but of some intrinsic factor associated with the presence of algae, since even during starvation green hydra produced more buds, survived longer, resisted disintegration and displayed a lower turnover of sulfur-labeled protein compared to aposymbiotic controls (Tables II, III). These results lend quantitative support to the observations of Goetsch (1924), who noted that (1) well-fed albino hydra grew as well as well-fed green individuals, either in light or in darkness, and (2) when starved in the light, green hydra survived for nearly twice as long as aposymbiotic controls. Partially "infected" hydra survived at least as long as any of the aposymbiotic controls and appeared "less depressed." Goetsch concluded that the algae were not essential when food was abundant but probably played some role in augmenting survival when certain stresses, *e.g.*, starvation, were imposed on the host. Karakashian (1963) demonstrated a positive influence of algae on growth of the ciliate protozoan, *Paramecium bursaria*, when bacterial food was present in low concentration and when cultures were starved but illuminated. A positive correlation of mean numbers of algae per paramecium with growth rate and survival time was also observed. Our results thus parallel these in several respects.

The adaptive value of symbiosis with algae is particularly evident from the behavior of albinos in these experiments. Their low budding rate and tendency to disintegrate early during starvation, and poor growth on a limited food supply would be disadvantageous for survival in an environment where limited food and periods of starvation are frequently encountered (Welch and Loomis, 1924). These observations perhaps explain why aposymbiotic adult *C. viridissima* have not yet been found in natural waters, although algae-free eggs are often produced by at least one strain of green and albino *C. viridissima* (unpublished observations).

Possible mechanisms by which the algae influence growth of the host

1. *Gas exchange and waste uptake*

Geddes (1882) suggested that symbiotic algae augment the well-being of their animal hosts in several ways, including (1) by taking up carbon dioxide and producing oxygen during photosynthesis, thus facilitating host respiration, and (2) by taking up host excretory wastes, such as ammonia, thereby creating a less toxic micro-environmental milieu for the animal. These interactions undoubtedly take place to some extent in most associations but as yet there is little direct evidence that any of them are essential to the animal (see Droop, 1963). In fact, they appear to be non-essential for *C. viridissima* since individuals without algae grow at k_{max} as long as they are well fed. Similarly, well-fed green and albino *C. viridissima* grow at nearly identical rates in darkness where photosynthetic gas exchange is again ruled out as an augmenting factor (Goetsch, 1924; our unpublished observations).

2. *Utilization of algal metabolic products*

As suggested by Geddes (1882) and others (Keeble, 1908; Boschma, 1925; Gohar, 1940, 1948) a host could benefit by digesting its symbiotic algae or utilizing their extracellular products. On the basis of the observations in this study, little can be said regarding digestion of algae by *C. viridissima*. Since there is no apparent decrease in number of algae after two to three weeks' starvation, and since 10–20% of the normal flora can sustain the starving host, digestion of algae seems unlikely but is not ruled out. As noted by Yonge (1944) symbiotic algae probably resist digestion since the majority are found in animals which display intracellular digestion.

However, there is evidence that *C. viridissima* utilizes products of algal metabolism. Experiments with $C^{14}O_2$ show that 10–20% of the labeled carbon fixed by the algae is transferred to the animal where some is incorporated into major chemical fractions (nucleic acids, proteins, etc.). The specific activity of C^{14} in algae-free green hydra tissues in these experiments was 50–100 times greater than that in albino control tissues where some carbon was assimilated solely by heterotrophic fixation (Lenhoff and Zimmerman, 1959; Muscatine and Lenhoff, 1963). Similar transfers take place in other coelenterate-algae associations (Muscatine and Hand, 1959; Goreau and Goreau, 1960) and are implied to occur in others (Sargent and Austin, 1949, 1954; Odum and Odum, 1955; Burkholder and Burkholder, 1960).

The results of this study and demonstration of the utilization of products of algal metabolism by host cells lend support to the conclusion that the algae in *C. viridissima* augment growth of the host by nutritional supplementation. Support for this view comes also from the observation that adequate food can replace the need for symbiotic algae (Goetsch, 1924; Fig. 2, this paper). A similar observation was reported by Parker (1926) and Karakashian (1963) for *P. bursaria.* The inability of albino *C. viridissima* to withstand starvation and the tendency to disintegrate undoubtedly reflects a loss of function by this species. Neither green *C. viridissima* nor the non-symbiotic species, *H. littoralis,* show this reaction to starvation, which could be symptomatic of a nutrient deficiency, as a result of a metabolic lesion. The growth lags and slow responses of albinos to intermittent feeding (Fig. 2) may represent the time needed to accumulate essential nutrients from the limited food supply. In contrast, green hydra did not exhibit extended growth lags or delayed responses to intermittent feeding. Auxiliary metabolites received from the algae probably offset any deficiency, though only temporarily, since, as shown in starvation experiments (Table II), the algae cannot sustain the animal indefinitely without some exogenous food. Information on carbon turnover rates by the algae, their extracellular products, the growth requirements of the host, and the peculiarities of the metabolism of algae-free individuals should bring to light the details of mechanisms of host-symbiont interaction in this association.

Part of this investigation was carried out at the Laboratories of Biochemistry, Howard Hughes Medical Institute, Miami, Florida during the tenure of a Postdoctoral Fellowship from the Division of General Medical Sciences, United States Public Health Service (1963) to Leonard Muscatine, and an Investigator Award of the Howard Hughes Medical Institute to Howard M. Lenhoff. We thank Mr. J. Bovaird, Mr. Alfredo Lopez, and Mr. Enrique Nagid for technical assistance.

NOTE ADDED IN PROOF

Slobodkin (1964) has recently demonstrated that the "ecological efficiency" (yield energy/food energy) of experimental populations of *C. viridissima* is about four times higher than that of *Hydra littoralis* (a non-symbiotic species), but only in populations grown in the light. The implication is that photosynthetic carbon is available to *C. viridissima* for energy. Slobodkin, L. B., 1964. Experimental populations of Hydrida. *J. Ecol. (Suppl.)*, **52**: 131–148.

SUMMARY

1. When fed daily on *Artemia* nauplii, green and albino *C. viridissima* grew at nearly identical logarithmic rates. With limited food, growth of green hydra always exceeded that of albinos. This difference was not the result of a quantitative difference in food intake.

2. Green hydra survived starvation for about four weeks, gradually diminishing in size. Albinos survived only 10–12 days, succumbing to starvation by relatively sudden disintegration.

3. The relationship between survival ability and algal content was non-linear. Animals with 20% of the normal flora survived nearly as well as those with a full complement of algae.

4. Turnover rate of sulfur-labeled protein during starvation showed the relationship albino > pale green > green, among the groups tested. The presence of symbiotic algae appears to depress the rate of protein catabolism.

5. It is concluded that symbiotic algae augment growth, budding, and survival of *C. viridissima* (Carolina strain 1960) by a mechanism which does not appear to involve gas exchange or waste removal by the algae.

6. Evidence is presented in support of the hypothesis that algal metabolic products augment growth and survival of *C. viridissima*.

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THE DEVELOPMENT OF EGGS OF THE SCREW-WORM FLY COCHLIOMYIA HOMINIVORAX (COQUEREL) (DIPTERA: CALLIPHORIDAE) TO THE BLASTODERM STAGE AS SEEN IN WHOLE-MOUNT PREPARATIONS

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In studying effects of radiation or chemical mutagens on insect germ cells, being able to examine the chromosomes of nuclei in freshly deposited eggs would provide many advantages. Meiotic division of the oocytes typically occurs after the eggs are deposited. Thus, both the meiotic nuclei and the mitotic ones of the cleavages or later stage divisions could be studied. In addition, only in freshly laid eggs can the chromosomes of sperm be directly examined after treatment that produces dominant lethal mutations. In spite of these advantages, only a few such studies have been made of the nuclei of very young eggs, to ascertain the presence of chromosomal aberrations or abnormal nuclear divisions and death (*i.e.,* Sonnenblick, 1940, on *Drosophila*; Whiting, 1945a, 1945b, von Borstel, 1955, on *Habrobracon*). Neglect of this kind of study has probably been due in part to technical difficulties in collecting and preparing enough eggs at the exact stage of development required. In addition, nuclei and chromosomes of such eggs, as a rule, are very small and thus difficult to study.

Recently the author and his associates became interested in determining whether chromosome aberrations induced in the sperm or oocytes of screw-worms, *Cochliomyia hominivorax* (Coquerel), could be studied in the young egg, in at least a reasonably satisfactory manner. Sectioned material was quickly found unsatisfactory for this purpose and emphasis was placed on whole-mount preparations. Normal development to the stage of blastoderm formation, as followed in the whole-mounts, is described herein. Chromosome aberrations actually found in young eggs after treatment of screw-worm flies with gamma radiation and the alkylating agent, tretamine, were discussed in an earlier paper (LaChance and Riemann, 1964).

MATERIALS AND METHODS

The sexually mature females of the Florida Normal strain used in this study usually oviposit readily when offered warm lean meat. Each female will then normally produce 200–300 eggs within a period of 15–20 minutes. These eggs, cemented together in a compact mass, are each about 0.7 mm. long and no wider than 0.16 mm. Each one is covered with a rather thick chorion which must be removed in making a whole-mount preparation.

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To obtain eggs of a fairly uniform age, individual flies were allowed to oviposit for one minute in shell vials placed in a water bath heated to 35° C. The flies were then discarded and the vials with the deposited eggs, usually 10 to 20 in number, were removed from the water bath and held at room temperature (24–26° C.) until fixation.

The whole-mount procedure was essentially that described by von Borstel and Lindsley (1959) in their modification of the technique developed by Schmuck and Metz (1931). First, the eggs were dechorionated by shaking them gently in 1% sodium hypochlorite (commercial bleach diluted 1:5 in distilled water) for not more than two minutes. By the end of this period most eggs had lost their chorions. The eggs were removed from the hypochlorite solution by straining them through a cloth. They were then washed briefly in distilled water and placed in rows on 22-mm. coverslips by means of an artist's brush. Each egg was then gently punctured (usually at the posterior or blunt end) with a fine needle so that a thin stream of ooplasm flowed onto the surface of the coverslip. This puncturing was essential for good fixation and the extruded ooplasm served to attach the eggs firmly to the coverslips. As soon as possible after puncturing, the coverslip with its attached eggs was placed in Kahle's fixative. All fluids used in preparing the eggs were kept at room temperature.

It took about 4–5 minutes to handle 10–15 eggs, from the begintning of dechorionation to placing the eggs in the fixative. Fewer eggs could be handled somewhat faster. In general, different groups of eggs fixed at comparable times after deposition were quite similar in stage of development. Also, puncturing one end of the egg or the other, or even allowing the nuclei to flow out of the egg, made little difference in the timing of the stages of development, since fixation occurred rapidly.

After fixation, the eggs were stained according to the Feulgen procedure with Schiff's solution, as outlined by von Borstel and Lindsley. Because of the eggs' rather considerable thickness, the stained and dehydrated specimens were usually mounted on another coverslip instead of on a slide. In this way the mounts could be easily turned over to permit examination of both sides under high magnification. Generally the preparations were allowed to clear for a few days in the mounting medium (Diaphane) before they were examined. This was particularly desirable for examining cleavage nuclei. An optical system that produced little contrast was also found to be highly desirable for examining eggs.

More than 600 eggs were examined in preparing this description of egg development in the screw-worm. Two series of eggs fixed at 15-minute intervals through the first two hours of development and one series fixed at 5-minute intervals from the first to the third hour were prepared. Also prepared were numerous other series of eggs less than one hour old, particularly less than 25 minutes old, since this time interval covered the most significant periods in determining the effects of mutagens on germ cells.

All drawings were made with a camera lucida. However, to save space, some nuclei were drawn somewhat closer together than they would have appeared in true scale.

Observations and Discussions

LaChance and Leverich (1962) demonstrated that the meiotic nuclei of screw-worm eggs go through prophase I and metaphase I while the eggs are still in the ovaries. By the time the eggs have reached maturity in the 5-day-old females, the oocytes are in early anaphase I, in which stage they remain until the eggs are actually deposited. The chromosomes (six pairs) of these anaphase nuclei are much contracted, and each meiotic figure appears to be merely a single, very small chromatin mass in which some of the homologous chromosomes probably remain in contact with each other. Each nucleus is located in the superficial ooplasm at a point on the dorsal surface of the egg about one-fifth of the total egg length from the anterior end.

In a few eggs, fixed individually as early as three minutes after deposition, the nuclei were still in the condensed early anaphase characteristic of the mature ovarian eggs. Nuclei 1–2 minutes older had longer chromosomes and were usually in late anaphase or telophase I (Fig. 1). After telophase I, the egg nuclei went at once, without any interphase stage, through the second meiotic division, which was completed in 7–8 minutes. At the end of telophase II (Fig. 2), all egg nuclei had formed a straight line that extended from the site of the original anaphase I nucleus to a point somewhat further into the interior of the egg but still near the surface. The position of the line of nuclei (and hence the division plane at anaphase I) with regard to the long axis of the eggs varied widely from egg to egg. In some eggs it extended posteriorly, in others anteriorly, and in still others in various intermediate positions, although always with one end located further in the ooplasm than the other.

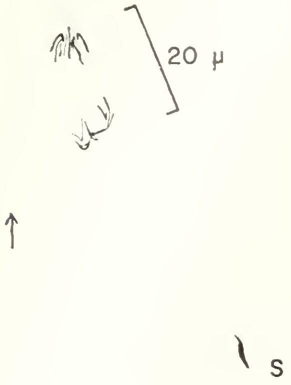
The chromosomes of the meiotic nuclei were extremely small and as a rule individual ones could not be recognized, even in the anaphase figures where they were usually fairly well separated.

Immediately after completion of the second division, the terminal nucleus (the one farthest inside the egg) swings out of line and moves as the female pronucleus to a central position in the egg interior at about the same level on the long axis of the egg as the original oocyte. As the female pronucleus moves, it is transformed into an interphase nucleus surrounded by a membrane. The other three meiotic nuclei remain in their original positions as the polar bodies. However, they quickly follow the pronucleus into interphase. This transition starts in the other terminal nucleus a little before it does in the two medial ones.

During the period when the meiotic divisions are taking place, the heads of such sperm as may be present (from 1–5), are seen as short Feulgen-positive rods (Figs. 1 and 2), usually located in the deeper ooplasm of the same general level as the oocyte nuclei. Occasionally a more elongate, threadlike sperm head, such as normal motile sperm possess, was noted, but usually only the short rods were seen, even in the youngest eggs. Sperm tails were presumably present in the eggs but they did not stain and could not be detected.

In near synchrony with the oocyte nuclei, all sperm heads in an egg were transformed into interphase nuclei. One of these nuclei moved to a position immediately adjacent to the female pronucleus to become the male pronucleus. Thus, by the end of about 9 minutes, only interphase nuclei were present in the eggs. When first formed, these nuclei have a diameter of about 5½ microns which increases to 12 microns. Presumably during this 9-minute period cell synthesis takes place, involving among other things DNA replication.

By the end of about 12 minutes, all nuclei, including the polar bodies and any extra sperm nuclei that might be present, have entered into early prophase (Fig. 3). By 14–15 minutes they have usually reached metaphase with the two pronuclei being included in a single unit to complete syngamy. The first cleavage division (Fig. 4) quickly follows and succeeding cleavages occur in rapid succession (Fig. 5). Approximately 5 minutes separate the first few cleavages, but the others occur somewhat less rapidly. By the end of one hour the eighth and final internal cleavage division is usually in progress. Thus, at 25°–26° C. cleavage within the interior of the egg occupies a period of about 45 minutes, with the average division cycle lasting only about 6 minutes. This rate is somewhat faster than the cleavage cycle of about 9–10 minutes at 20°–30° C. reported for *Drosophila* (Sonnenblick, 1950) and 10



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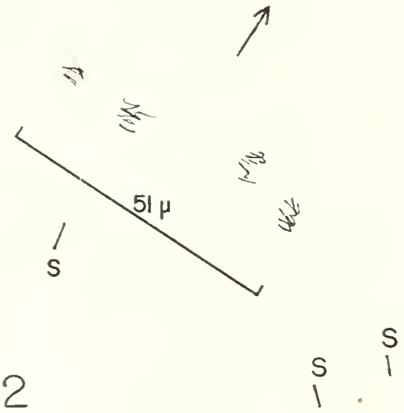

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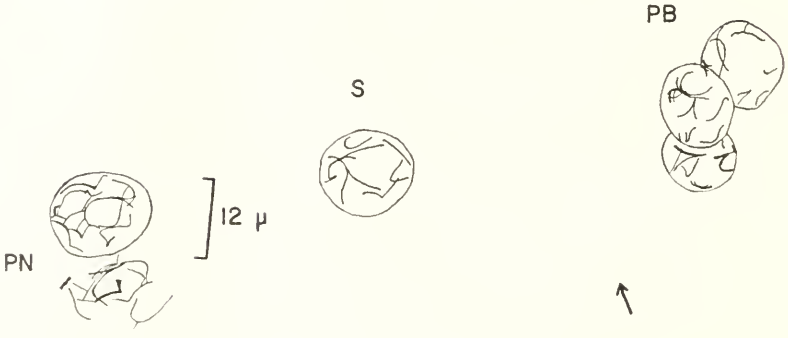

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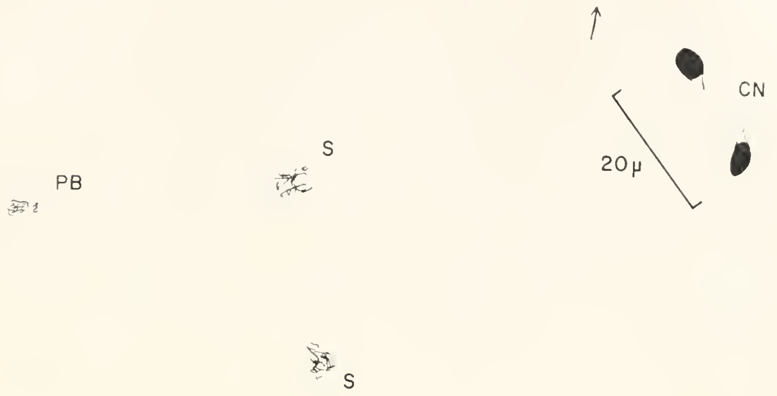
Figure 1. Four–5-minute egg, telophase I.

Figure 2. Seven–8-minute egg, telophase II.

Figure 3. Eleven–12-minute egg, late prophase before syngamy. The nuclear membrane had disappeared from one of the pronuclei.

minutes at 20° C. for *Calliphora vicina* (R. and D.) [*erythrocephala* (Meigen)] (Melander, 1963) and may represent the most rapid rate of mitosis reported for multicellular animals (Mazia, 1961), although it would seem rather probable that other higher Diptera develop as rapidly.



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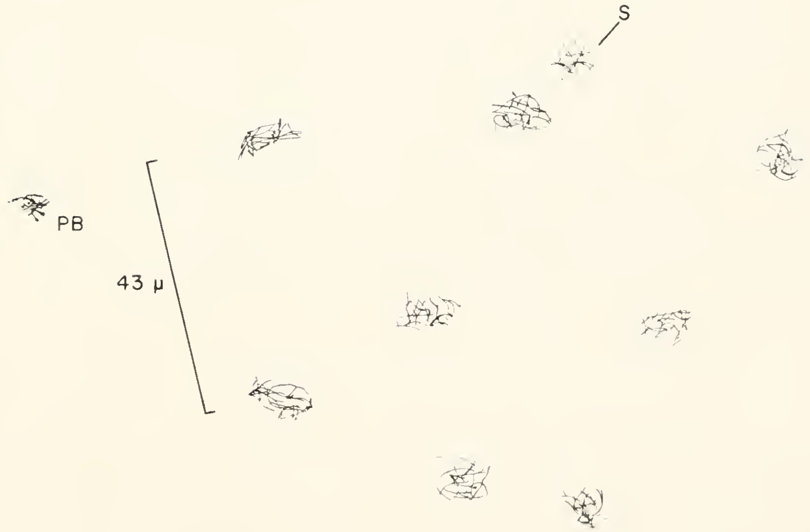

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FIGURE 4. Fifteen–16-minute egg, first cleavage telophase.
FIGURE 5. Thirty–31-minute egg, fourth cleavage metaphase.

Observing cleavage nuclei was rather less satisfactory than observing meiotic ones. In part, the difference was due to the obstruction caused by the overlying ooplasm even after it had cleared, but in addition the cleavage nuclei appeared to stain less deeply. Again, individual chromosomes could not be distinguished as a rule. Sometimes male and female chromosome complements remained somewhat separated during the first cleavage division, but more usually they could not be recognized after they had formed the first metaphase figure. Viewing cleavage divisions was made easier by the fact that they all occurred in a plane rather closely parallel to the egg surface.

The first cleavage nuclei remain in the area in which syngamy occurs. With repeated divisions there is a spread of nuclei, and by the end of the eighth division they are rather evenly distributed along the length of the egg.

The somewhat slower velocity of the later internal cleavages appeared to be associated with a change in duration of the different stages of cell division. Very careful timing was required to obtain metaphase, anaphase, or telophase figures during the first 2–3 cleavage divisions. On the other hand these stages were found more often than interphases or prophases in eggs fixed at various later times. All the first 8 cleavages are probably completely synchronous in the intact egg, but in the usual stained specimen a slight gradient of development away from the site of puncturing was noted.

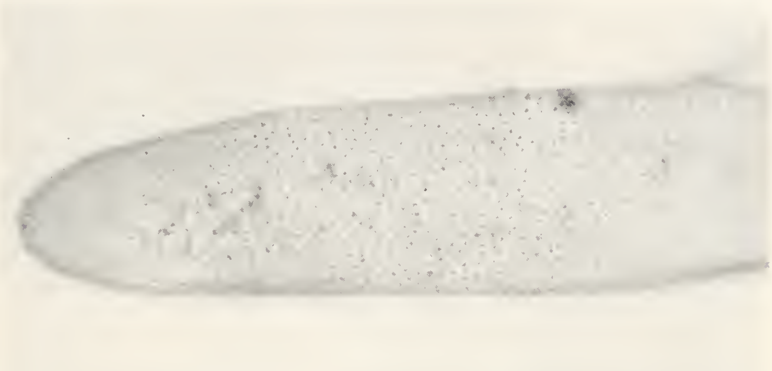
After the end of the eighth cleavage division in the interior of the egg, most nuclei have migrated near the surface of the egg to form the incipient blastoderm or blastema by the end of 1 hour and 10 minutes. After the blastema is first formed, four other cleavage divisions of most of the nuclei occur. The first of these divisions takes place shortly after the nuclei complete their movement to the egg surface. The last division is usually underway or completed by the end of the second hour. This cycle, lasting about 15 minutes, compares rather closely with the 17-minute cycle that Agrell (1963) and Melander (1963) reported for the same four divisions in *C. vicina*. After the 12th division a prolonged interphase takes place, at the end of which further divisions occur. During the long interphase period, cell membranes are formed around the nuclei located in the surface ooplasm, to become the definitive blastoderm.

These last four cleavages are not completely synchronous like the first 8, but instead appear to follow the pattern reported by Agrell (1963) for *C. vicina*. For the first three divisions there was a rather slight mitotic gradient from the anterior end of the egg, and also during the 11th division some nuclei near the posterior end were observed to divide earlier than those more anterior. During the 12th division a definite mitotic gradient proceeding from both ends of the egg was noted (Fig. 6).

Not all of the early cleavage nuclei migrate to the surface ooplasm to form the blastema. Some remain behind as the so-called yolk nuclei or vitellophags. These divide out of synchrony with the blastema nuclei, to form rather massive clusters of nuclei (Fig. 7) by the time the 12th division is completed. No evidence was seen to indicate that any of the blastema nuclei migrate inward to increase the number of yolk nuclei.

Shortly before the 10th cleavage a cluster of nuclei appear outside of the blastema at the posterior end of the egg. These are the so-called pole cells which presumably, here as in other species, include the primordial germ cells. In *C. vicina* these cells (actually only nuclei) are also set off at about the time of the 10th division, although, according to Sonnenblick (1950), the first ones appear at the time of the 9th division in *Drosophila*. No divisions were detected among the pole cell nuclei during the periods when the later cleavages occurred.

Melander (1963) described in great detail how the "pseudo-chiasmata," formed during the 9–13th nuclear divisions in *C. vicina,* result in chromosome diminution by causing the loss of small chromosome fragments. No attempt was made to study possible chrosome diminution in the screw-worm. However, in the blastema many anaphases exhibited configurations that appeared to be similar to the pseudo-chiasmata described by Melander. Thus, it seems probable that the events in the two species are at least somewhat similar.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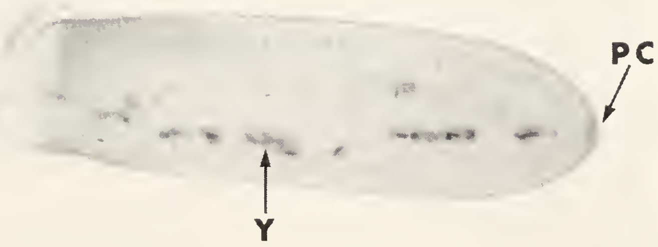

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FIGURE 6. One hour and 59–60-minute egg. The nuclei at either end have gone into interphase while those in the central region are still dividing; 400 ×.

FIGURE 7. Two hour and 15–16-minute egg, showing the clumped yolk cells and the interphase nuclei of the blastema after the completion of the 12th division; 320 ×.

Explanation of captions: C N, cleavage nuclei; P B, polar bodies; P C, pole cells; P N, pronucleus; S, sperm cell; Y, yolk cells.

As stated earlier, the polar bodies and surplus sperm nuclei of screw-worm eggs go into metaphase at the same time as the pronuclei and remain in this stage until they eventually disappear. The sperm nuclei can be seen during early cleavages

TABLE I

*Time sequence in the development of screw-worm eggs through blastoderm formation**

Minutes	Stage of development
0–4	Sperm move into area of oocyte which remains in early meiotic anaphase I.
4–6	Meiotic division I is completed.
5–8	Meiotic division II is completed.
8–12	All nuclei go into interphase. Pronuclei move to adjacent position in the interior of the egg.
12–13	All nuclei pass synchronously into metaphase. Syngamy occurs as the two pronuclei form a single figure.
14–16	Completion of cleavage I.
29–31	Cleavage III in progress or completed, 4–8 nuclei.
44–46	Cleavage V completed and division VI may be in progress; 32 nuclei.
59–61	Cleavage VIII generally in progress; 128 dividing nuclei.
74–76	Blastema formed with division IX usually in progress.
90–91	Division X in progress. Pole cell nuclei set aside.
104–106	Division XI usually in progress.
120–122	Division XII usually in progress.
150	Cell membranes around surface nuclei, definitive blastoderm.

* Oviposition at 35° C., development after one minute at 24–26° C.

but not during the later ones. The polar bodies remain until the blastema is formed but then quickly undergo dissolution. Very rarely we observed one of the haploid polar bodies dividing at the same time as the first cleavage nucleus.

2. *Polyspermy in screw-worm eggs*

It has long been considered a general rule that in insects each egg is penetrated by several sperm (Wigglesworth, 1950). However, Hildreth and Lucchesi (1963) found that, contrary to the observations of earlier workers, the eggs of *Drosophila melanogaster* and *D. virilis* usually received only a single sperm. As a result of their studies, they raised the question as to how common polyspermy might actually be in insects.

Many screw-worm eggs contained only a single sperm, but more often than not they had two or more sperm. In no egg, however, were more than 5 sperm found and this number was quite unusual. Also, an inseminated fly often deposited a few eggs that had not been fertilized. The presence of these unfertilized eggs probably explains why somewhat less than 100% of the eggs from normally inseminated females usually hatch.

TABLE II

Distribution of sperm found in a sample of screw-worm eggs

Unfertilized eggs	2
Eggs containing one sperm	30
Eggs containing two sperm	39
Eggs containing three sperm	14
Eggs containing four sperm	3
Total number of eggs	88
Total number of sperm	162

The distribution of sperm in 88 eggs from 10 different females is shown in Table II. All of these eggs were fixed before they were 8 minutes old, when the rodlike sperm heads are relatively easy to count.

Occasionally a sperm nucleus was observed very near one of the polar bodies although in no instance had a second zygote actually been formed. However, the appearance of an occasional gynandromorph probably indicates that a second zygote is sometimes formed.

3. *Development of unfertilized eggs*

Virgin screw-worm females oviposit nearly as readily as inseminated ones, but their eggs apparently never hatch. However, young eggs in which no sperm could be detected often completed both meiotic divisions. To determine how far development advances in unfertilized eggs, whole-mounts of 8–20 eggs were prepared from each of 15 virgin flies. One portion of the eggs were fixed at 5½–7 minutes after deposition, another portion at 14–16 minutes, and a third at 1¼–2 hours. To eliminate any possibility that dechorionation and other steps involved in the handling of the eggs might initiate development, these operations were limited to not more than 5 minutes immediately prior to fixation.

TABLE III

The development of eggs from virgin females

	Age at fixation		
	5½–7 min.	14–16 min.	1¼–2 hrs.
No. of females	5	6	4
No. of eggs	53	72	60
Aborted meiotic division I	12		
Meiotic division I completed or in progress	19		
Meiotic division II completed or in progress	22		
Pronucleus not formed or did not migrate to interior of egg		35	33
Normal migration of pronucleus		33	24
Cleavage I completed or in progress		4	3
Cleavage II completed or in progress		0	0

Results of observations on the stained preparations are shown in Table III. They demonstrate that fertilization is not strictly necessary for initiation of development. However, it is also evident that the presence of sperm in the eggs has some influence even on meiotic divisions, for nearly one-third of the nuclei fixed at 5½–7 minutes had failed to complete the first division. It was also obvious that some of the other nuclei could not have gone through the second division. Even in eggs in which the two divisions were completed, the pronuclei had often failed to migrate into the interior of the egg. Thus, in over half the eggs fixed at 14 minutes or later, clumps of chromatin were found only at the site normally occupied by the three polar bodies. In some eggs, each of these clumps was observed to be composed of four smaller clumps representing all of the meiotic nuclei. Observations on other unfertilized eggs fixed at 8–9 minutes demonstrated that when meiosis was completed, the oocyte nuclei went through the usual interphase stage before returning to metaphase.

Scoring of the older unfertilized eggs was easier because development always stopped at a stage at which all nuclei were in metaphase. Apparently no nuclei had disappeared by the time the oldest preparations were fixed.

The author wishes to express his appreciation to Dr. Leo LaChance, who suggested this study and helped in many ways during its progress. He also wishes to thank Miss Ann Leverich and Miss Sarah Bruns for their technical assistance.

Summary

1. Development of screw-worm eggs from the first meiotic division to blastoderm formation was studied from whole-mount preparations. Both meiotic divisions were completed by 7–8 minutes. Syngamy at 14–15 minutes was quickly followed by the first cleavage division. The first 8 cleavages took place within the interior of the eggs, and the last of these occurred approximately one hour after egg deposition. After the 8th division, most cleavage nuclei moved near the egg surface to form the blastema by the end of about 1 hour and 10 minutes. This movement was followed by four more divisions of the blastema nuclei. The last of these divisions was underway, or had been completed, by the end of the second hour. There was then a prolonged interphase period, during which cell membranes formed around the blastema nuclei to become the definitive blastoderm.

2. The first 8 cleavages were synchronous, but for the last four divisions an anterior-posterior gradient was evident. During the 11th division, and especially during the 12th division, there was also an accompanying mitotic gradient proceeding from the posterior end of the egg.

3. After the 8th cleavage some nuclei remained behind to form the yolk nuclei or vitellophags. Also, the pole cells were set aside after the 9th division but prior to the 10th.

4. A low order of polyspermy was found in most eggs, but many of them received only a single sperm. Most unfertilized eggs completed at least the first meiotic division, but very few of them achieved the first cleavage division and none developed further than this stage.

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THE RELATIONSHIP OF SALINITY TO LARVAL SURVIVAL AND DEVELOPMENT IN NASSARIUS OBSOLETUS (GASTROPODA)[1, 2]

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Not until recently has the relationship of salinity to the survival and development of marine larvae been seriously examined through laboratory observations. The earliest studies upon mollusks were confined almost exclusively to the embryonic and very early shelled pelagic stages of pelecypods. Thus, Seno, Hori and Kusakabe (1926), Amemiya (1926), and Rao (1951) determined the effect of reduced salinities upon the early development of, respectively: *Ostrea gigas* and *Crassostrea virginica; Ostrea angulata* and *Ostrea edulis;* and *Ostrea madrasensis.* Because the early larvae of *Crassostrea virginica* survived salinities far lower than those found in the habitat of the adult, Clark (1935) concluded that the effect of salinity was unimportant in determining the mortality of oyster spat in Malpeque Bay, P.E.I., Canada. Turner and George (1955) showed experimentally that the larvae of *Venus mercenaria* would not swim past a salinity discontinuity of 20 and 15 parts per thousand (‰). The very interesting results of Haskin (1964) reveal a direct relationship between salinity and swimming activity of oyster larvae (*Crassostrea virginica*). Haskin also demonstrated that light intensity and its spectral composition modify the response elicited from late "eyed" oyster larvae. Wells (1961) has compared the "salinity death points" of the adults and early larvae of two species of gastropods, *Thais floridana* and *Cerithium floridanum.* In the former, little difference between the "salinity death point" of the adult and larva was found, whereas in the latter species the "salinity death point" of the larva was at a higher salinity than of the adult. Not until the investigations of Davis (1958) upon *Crassostrea virginica* and *Venus mercenaria* and of Davis and Ansell (1962) on *Ostrea edulis* have observations on the effect of salinity over the entire pelagic period of larval molluscan development been made. Stickney (1964) in addition has recently cultured *Mya arenaria* larvae and noted their response to salinity. No laboratory experiments on the relationship between salinity and growth in estuarine prosobranch gastropod veliger larvae have been published. Field studies on the effect of salinity upon survival and development of larval mollusks deal largely with commercially important species (Nelson and Perkins, 1930; Carriker, 1951; Korringa, 1952; Kunkle, 1957; Haskin, 1964; etc.) and no attempt to summarize this work is made here.

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A common species which inhabits the intertidal flats of estuaries along the east coast of North America from Chaleur Bay in the Gulf of St. Lawrence to northern Florida is the mud snail or basket shell, *Nassarius obsoletus* Say. The ecology, certain aspects of which have recently been reviewed (Scheltema, 1964), is rather well known and the pelagic larval development and early post-larval life history have been described (Scheltema, 1962a). The lower limits of salinity at which the adults of *N. obsoletus* are naturally found range between 15‰ and 20‰.

Though much is known about this ubiquitous species, nothing has yet been reported on the effect of salinity upon survival and growth of the veliger larvae. I report here upon the results of some experiments with the larvae of *N. obsoletus* which (1) demonstrate the lower limit at which salinity becomes lethal to both the larvae and adults, and (2) show the effect of reduced salinity on growth between the time of emergence from the egg capsule to the completion of larval development.

Experiments on the Lower Lethal Salinity for Nassarius obsoletus

The lethal salinity for a species may be determined either experimentally in the laboratory or from observations in the field. In the laboratory, organisms may be subjected to different salinities and their behavioral or physiological responses measured (*e.g.*, Blum, 1922), while in the field unusual natural conditons, such as sudden changes in runoff, may, by large mortalities, show when salinity limits tolerated by a particular species have been surpassed (*e.g.*, Beaven, 1946).

A criterion to be used in the laboratory by which the effect of salinity upon an organism may be quantitatively measured is difficult to find. The methods adopted here were unsophisticated, but the results were reasonably reproducible.

Salinity lethal to adult snails of *N. obsoletus* was determined by placing either 15 or 20 organisms from a collection made on the intertidal flats in one of a series of four-liter tanks. Salinities in the series systematically descended in value from full-strength sea water to about 5‰. Reduced salinities were obtained by diluting sea water with tap water. The resulting salinity in each tank was checked with a hydrometer. The interval between tanks in the initial experiments was 5‰. In subsequent experiments this was reduced to 2‰ as values approached the lethal limit of the organisms. All the experiments were performed at room temperature (*ca.* 20° C.). The animals were collected from a brackish-water estuary near the laboratory and held overnight at a salinity of 18‰ before use. At the beginning of the experiment the snails were directly transferred to the salinity being tested. The effect of salinity was appraised by watching the behavior of the snails. Snails completely withdrawn into the shell were considered "inactive," whereas those not completely withdrawn were regarded as "active." At each observation the percentage of active and inactive snails was recorded.

The results of these simple experiments are shown in Figure 1. The graph illustrates that the region of stress lay between 12.5‰ and 13.5‰ salinity. The results are after four hours, but when the experiments were extended over a period of three days the values do not differ significantly. A slight increase in the percentage activity was evident, but the lower lethal limit was not markedly shifted, nor was the value at which stress was first observed altered.

The lower lethal limit of salinity among veliger larvae of *N. obsoletus* was determined by methods similar to those used for the adult snails. The criterion used to determine the effect of salinity upon the larvae was their swimming activity. Fifty larvae shortly after their emergence from the egg capsule were placed in 400 ml. of sea water, the salinity of which was adjusted by the addition of tap water. The salinities tested ranged between 8.5‰ and 33.0‰ and were spaced at intervals of roughly 6‰ in the earlier and 3‰ in the later experiments. The temperature throughout was between 25° and 26° C., which is near the optimum for growth.

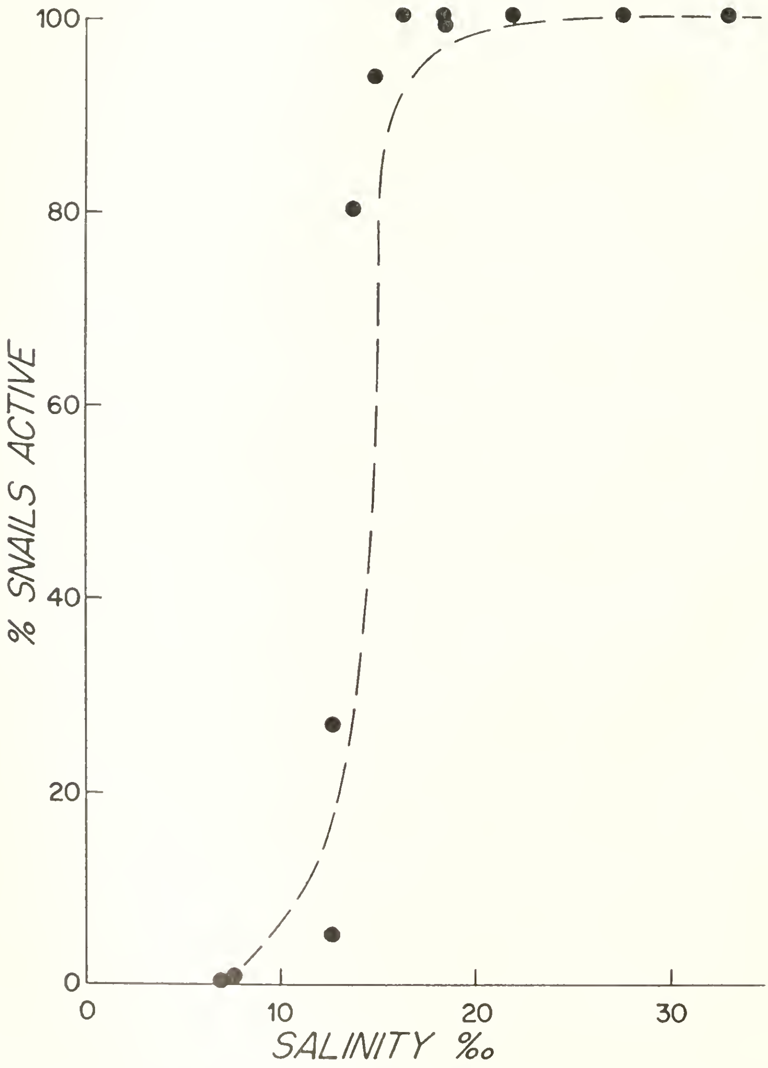

FIGURE 1. Inhibition of activity in adult *Nassarius obsoletus* resulting from reduced salinity at about 20° C. The graph illustrates the percentage snails active following a four-hour exposure period.

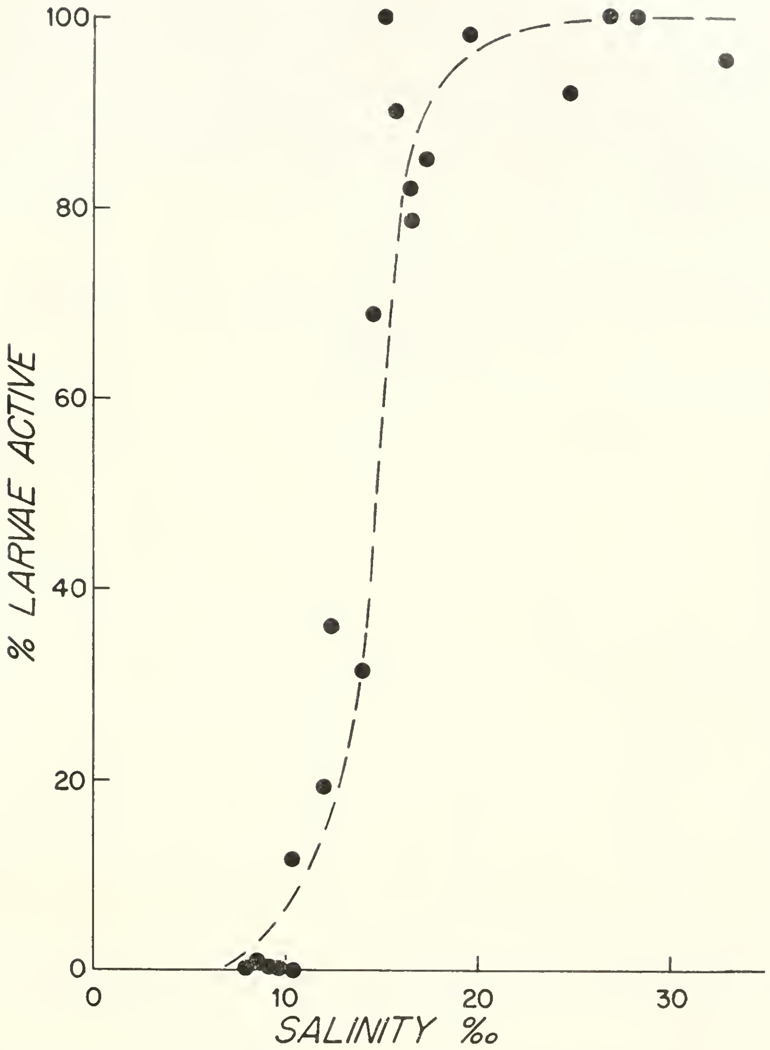

FIGURE 2. Inhibition of swimming in larvae of *Nassarius obsoletus* resulting from reduced salinity at 25–26° C. The larvae are those taken shortly after their emergence from the egg capsule. The results are following a 10-hour exposure period.

Larvae for all the experiments originated from water of 32‰ salinity. At the end of 10 hours the dish containing the larvae was placed under a bright light and those swimming were counted. The results of these experiments are shown in Figure 2. The greatest decrease in the percentage of swimming larvae falls between 14‰ and 15.5‰ salintity. Below 10‰ no larvae were ever seen swimming after the 10 hours of exposure.

The response of the late stage creeping-swimming larva of *N. obsoletus* was compared with that of the early veliger just after its emergence from the egg capsule.

These further experiments differed only in detail from those already described. Five-centimeter petri dishes were filled with dilutions of sea water ranging from 6.6‰ to 33.0‰ and in each, 10 veligers were placed. Into one sequence of dilutions were pipetted the early larvae; into the other, veligers which had completed their development to the creeping-swimming stage. The latter were reared in the laboratory for 19 days at 24° C. according to the method already described (Scheltema, 1962a).

TABLE I

Activity indexes of Nassarius obsoletus veliger larvae as a function of salinity*

Early pelagic larvae
(1–3 days after emergence from egg capsule)

Salinity	1 min.	20 min.	1 hour	3 hours	Mean for 3 hours
6.6	0	1	0	0	0.3
9.0	0	0	1	1	0.5
11.0	0	1.5	1	2	1.1
13.2	0	2	2	3	1.8
16.5	1	3	3	3	2.5
19.8	3	4	4	4	3.8
26.4	4	4	4	4	4.0
33.0	4	4	4	4	4.0

Creeping-swimming stage**

Salinity	1 min.	20 min.	1 hour	3 hours	Mean for 3 hours
6.6	0.0	0.0	0.0	0.0	0.0
9.0	0.4	0.0	0.0	0.0	0.1
11.0	0.4	0.0	0.0	1.0	0.4
13.2	0.0	0.4	0.2	2.6	0.8
16.5	3.0	2.8	3.4	3.9	3.3
19.8	3.0	2.0	3.8	4.0	3.2
26.4	4.0	4.0	3.9	3.5	3.9
32.0	4.0	4.0	3.9	3.5	3.9

* The definition of this term is given in the text.

** The values in these experiments were based on evaluation of each individual larva's performance, while those of the early larvae were simultaneously estimated by assigning a value to all the larvae in the dish.

The results of these experiments were expressed in terms of "activity indexes" which were recorded at the beginning of the experiment and after 20 minutes, one hour and three hours. The following numerical values were used to describe the responses of the larvae and to compute the "activity indexes": 0, no movement of velar cilia; 1, cilia of velum moving but not vigorously enough to allow the larva to swim; 2, larva moving sluggishly along the bottom and sides of dish or, if creeping-swimming stage, then responding immediately to the touch of a pin; 3, actively swimming or creeping; and 4, very actively swimming or creeping. All examinations were made under bright light.

Results of one such experiment are summarized in Table I. A mean activity index of between 2.5 and 4.0 indicates the "normal" range of behavior. The activity indexes of the new and old larvae as a function of salinity have been plotted together in Figure 3. This graph shows that there is no significant difference in the activity of the two ages of larvae relative to the salinity. A sharp decrease in activity occurred between 13.5‰ and 16.5‰ salinity. Similar experiments of longer duration (38 hours) fully confirmed the results shown here.

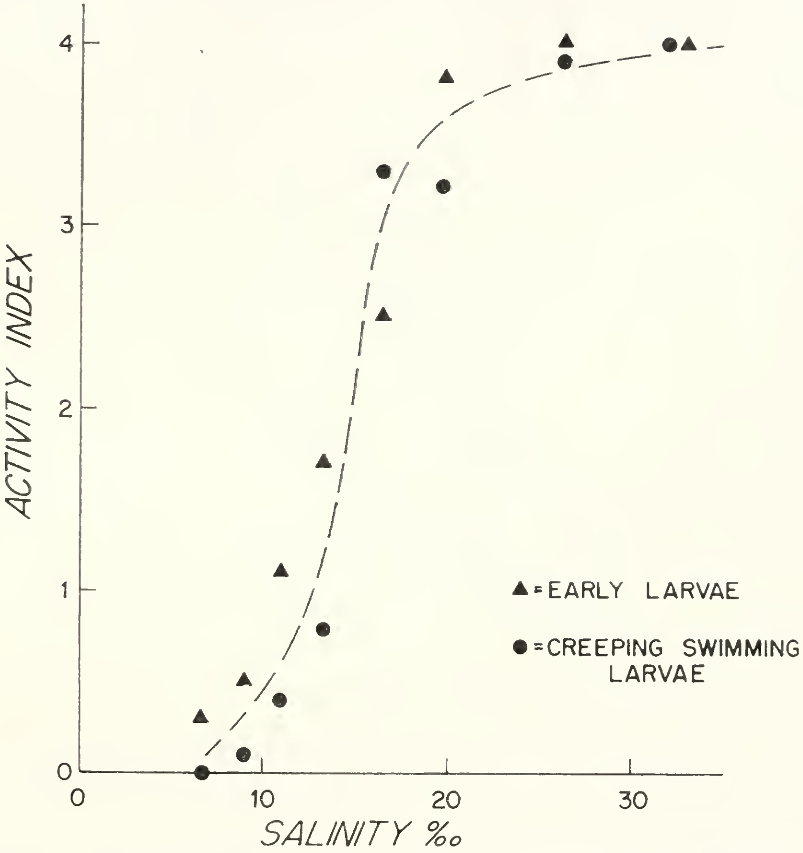

FIGURE 3. Relationship of the "activity index" of early and creeping-swimming larvae of *Nassarius obsoletus* to salinity. The graph illustrates the average "activity index" resulting from a series of observations over a period of three hours.

EXPERIMENTS ON THE EFFECT OF SALINITY UPON LARVAL GROWTH OF NASSARIUS OBSOLETUS

The larval life of *Nassarius obsoletus* can be divided into two periods. The first of these is a phase of rapid growth and external development leading to the creeping-swimming or veliconcha stage (Scheltema, 1962a). During this period the growth rate is essentially constant (Fig. 4). This is followed by a second period of very slow growth and no apparent further external morphological change. The beginning of the second period is evident from the completion of the development of the foot and from the behavior of the larvae, namely, frequent creeping on, and inspection of, the bottom .

The length of the first period is determined by those conditions which control larval growth.The length of the second period varies greatly, at least two-fold in *N. obsoletus,* and depends upon the encounter by the larva of a sediment suitable for post-larval life (Scheltema, 1961). Under favorable conditions metamorphosis may occur very near the beginning of the second period. The total length of

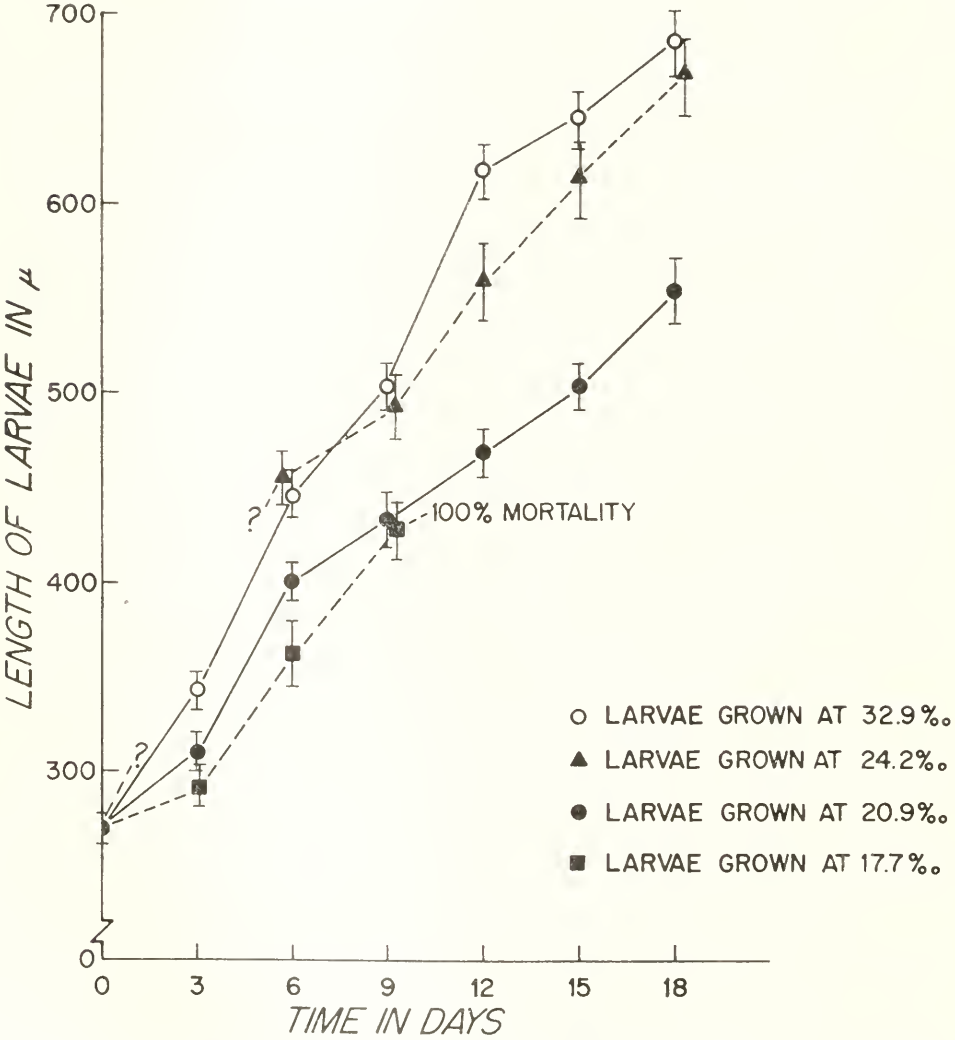

FIGURE 4. Increase in length of *Nassarius obsoletus* veliger larvae from the time of emergence from egg capsules to the completion of their development to the creeping-swimming stage. This is the first pelagic phase, during which growth occurs at a rapid and nearly constant rate. Each curve shows the result at a different salinity as indicated by the conventions.

larval life consequently is not determined solely by the growth rate, but also by the opportunity for metamorphosis upon a favorable substratum. In investigating the effect of salinity on the larvae, I have confined my attention only to the period of rapid and constant growth rate.

Laboratory experiments on the growth rate of veliger larvae are possible only after techniques for their mass culture are worked out (Scheltema, 1962a). The veliger larvae of *N. obsoletus* were grown in a series of 10-liter vessels with salinities ranging from that minimal for survival to that of full-strength sea water. It was soon found that the larvae held at a salinity of less than 16‰ did not survive more than a few days. Consequently the minimum average salinity in the experiment reported here was 17.7‰ and the remainder of the series had salinities of 20.9‰, 24.2‰, 26.2‰ and 32.8‰. All larval cultures were fed the euryhaline diatom, *Phaeodactylum tricornutum,* from the same algal culture.

TABLE II

Length in microns of Nassarius obsoletus throughout larval development as a function of the salinity

Mean salinity ‰	Age in days						
	0	3	6	9	12	15	18
17.7	278 ± 4*	291 ± 4	363 ± 8	429 ± 7	—	—	—
20.9	278 ± 4	312 ± 6	401 ± 6	433 ± 7	468 ± 6	501 ± 6	552 ± 8
24.2	278 ± 4	—	458 ± 7	492 ± 8	556 ± 9	613 ± 10	665 ± 10
26.2	278 ± 4	—	447 ± 6	487 ± 9	543 ± 8	586 ± 6	—
32.9	278 ± 4	343 ± 4	446 ± 5	502 ± 8	615 ± 8	643 ± 8	683 ± 9

* One standard error is indicated on all values of this table.

The mean temperature of the veliger cultures during this experiment, which extended over a period of 18 days, was 23.1° C. The maximum difference in water temperature measured during the course of the experiment was 1.1° C. However, since all cultures were kept together in the same temperature bath, the same fluctuations were experiencd by all. Maximum temperature differences between cultures were 0.5° C., but the mean difference was only 0.2° C.

At the lower values, the salinity in the cultures never varied more than ±0.5‰ from the mean; in cultures at salinities higher than 24‰, the maximum deviation from the mean value never exceeded ±0.7‰. Growth of the larvae was determined by measuring the maximum shell dimensions at three-day intervals with an ocular micrometer at a magnification of 100×. This measurement, hereafter termed *length,* was made on an aliquot of 30 larvae from each culture. Previous measurements have shown this to be an adequate sample size.

The results of the experiment (Table II) show that among salinities above 24‰ there is no statistically significant difference in length. As the salinity reached 21.0‰, the difference becomes significant. Below 21.0‰, the data indicate substantial decrease in shell growth rate. Under the conditions of the experiment, completion of larval development to metamorphosis did not occur at a salinity of 17.7‰.

The pertinent data are summarized by means of growth curves in the graph of Figure 4. Here the vertical lines indicate two standard errors. The difference between the two curves above 21‰ with those of 21‰ and less is quite evident, and the statistical significance is conspicuous.

The results of two additional experiments, similar to the one just described above, together with the data from the first experiment, are summarized in Table III. In each of the additional experiments, two cultures of veligers were started simultaneously with larvae obtained randomly from the same collection of egg capsules. The initial size of the larvae in the two cultures of an experiment were consequently the same. Development previous to emergence of larvae from the egg capsules was at room temperature. The unusually small initial size of the larvae in experiment III is a peculiarity of that particular collection of egg capsules and is probably related to the time at which they were deposited within the breeding cycle of the female snails.

Table III

Percentage inhibition of growth in Nassarius obsoletus larvae resulting from the lowest salinity at which development is completed to metamorphosis

Expt. no.	Temp. range °C.	Age of larvae at end of expt.	Length μ at begin expt.	A			B			Percentage inhibition (I)
				Mean salinity ‰	Length μ at end expt. (A)	Total growth* (ΔA)	Mean salinity ‰	Length μ at end expt. (B)	Total* growth (ΔB)	
I	23.0–24.0	18	278	32.9	683±9***	405	20.9	552±8	274	32.1
II	19.5–21.3	19	270**	33.1	701±4	431	21.5	634±6	364	15.5
III	26.1–28.0	12	249	33.2	545±5	296	21.3	504±8	255	13.8
									Sum	61.4
									Mean	20.5

* This denotes the difference between the initial length at the time of emergence from the egg capsule and the length at the end of the experiment.

** In this experiment only the length of the larvae at the termination of the experiment is known; the assumed length of 270 μ is the usual length of larvae at the time of emergence from the egg capsule. However, even if extreme values are assumed, the percentage difference in the final column is altered by no more than ±1%.

*** One standard error is indicated.

In one of the two cultures in each experiment, larvae were grown at the salinity of normal sea water. This culture is designated as "A" in each of the experiments of Table III. In the other culture, designated "B" in Table III, the larvae were grown at a reduced salinity near 21‰. Both cultures in each experiment were terminated at the same time. This was done near the end of the period of constant growth rate in the high salinity culture "A" of each experiment.

The percentage inhibition, I, due to the reduction of salinity is shown for each experiment in the right-hand column of Table III and was computed by the relationship

$$I = \frac{\Delta A - \Delta B}{\Delta A} \times 100,$$

where ΔA is the change in length of shell between the beginning and end of the experiment among larvae maintained at sea-water salinities, and ΔB is the change in shell length of larvae held at a minimum salinity required for completion of development. Table III shows that maximum inhibition was obtained at a temperature range of between 23° and 24° C., which is near the optimum for growth of the larvae of *N. obsoletus* (Scheltema, 1963). At both higher and lower temperature ranges there was substantially less inhibition in growth. However, owing to the large differences in larval growth rate frequently obtained between experiments, a direct effect or interaction of temperature on growth inhibition by reduced salinity cannot be assumed without further experiments. The results show that at the lowest salinity at which development to metamorphosis was completed, an average of 20.5% inhibition of growth occurred.

Discussion

Salinity as a limiting factor to distribution

The upstream distribution of most organisms that live within estuaries is seemingly related to salinity. However, mere correspondence between salinity values and the distribution of a particular species cannot in itself be taken as sufficient evidence that salinity is limiting. To show this, it is necessary to discover the extremes tolerated by an organism *throughout* its life history.

Thorson (1946, p. 472) has suggested that the larval stage may limit the distribution of bottom species, as this stage is "the weakest link of the chain." Experiments upon the salinity lethal to larval and adult *N. obsoletus,* however, showed no large difference between their lower tolerances. Such differences which did appear might be accounted for by the previous acclimation of the adult snails to a lower salinity or by the inadequacy of the techniques in making such small distinctions. The lower lethal salinity did not change significantly as larval development progressed (Figs. 2 and 3).

The known upstream distribution of *N. obsoletus* into estuaries in many instances seems to correlate well with the lower lethal salinity determined in the laboratory. Hence, Pfitzenmeyer (1961) found the species at locations in Chesapeake Bay where the summer salinity was 14.6‰. On the other hand, in certain estuaries on Cape Cod snails do not ascend farther up than a summer bottom salinity of 17‰, and in such instances salinity is probably not the factor limiting distribution. In order to make valid comparisons, laboratory results must be related to *bottom* salinities in areas where the seasonal extremes are known.

Salinity and larval growth rate

Growth of *N. obsoletus* is inhibited only as the lower limit of salinity tolerance is approached (Scheltema, 1962b). Thus, it was not until the salinity was near 20‰ that any significant effect on growth rate was noticed. Although not directly investigated, the decreased rate of growth in *N. obsoletus* is not likely to be related in any simple way to osmotic activity because marine mollusks, insofar as known, have no active osmotic control involving the expenditure of energy (Prosser *et al.*, 1961). The mortality of larvae at salinities below 20‰ was high.

No larvae survived beyond the intermediate stage of development in laboratory culture, although growth proceeded up until the ninth day at 17.7‰ salinity. The inhibition of growth attributable to the reduction of salinity amounted on the average to about 20%.

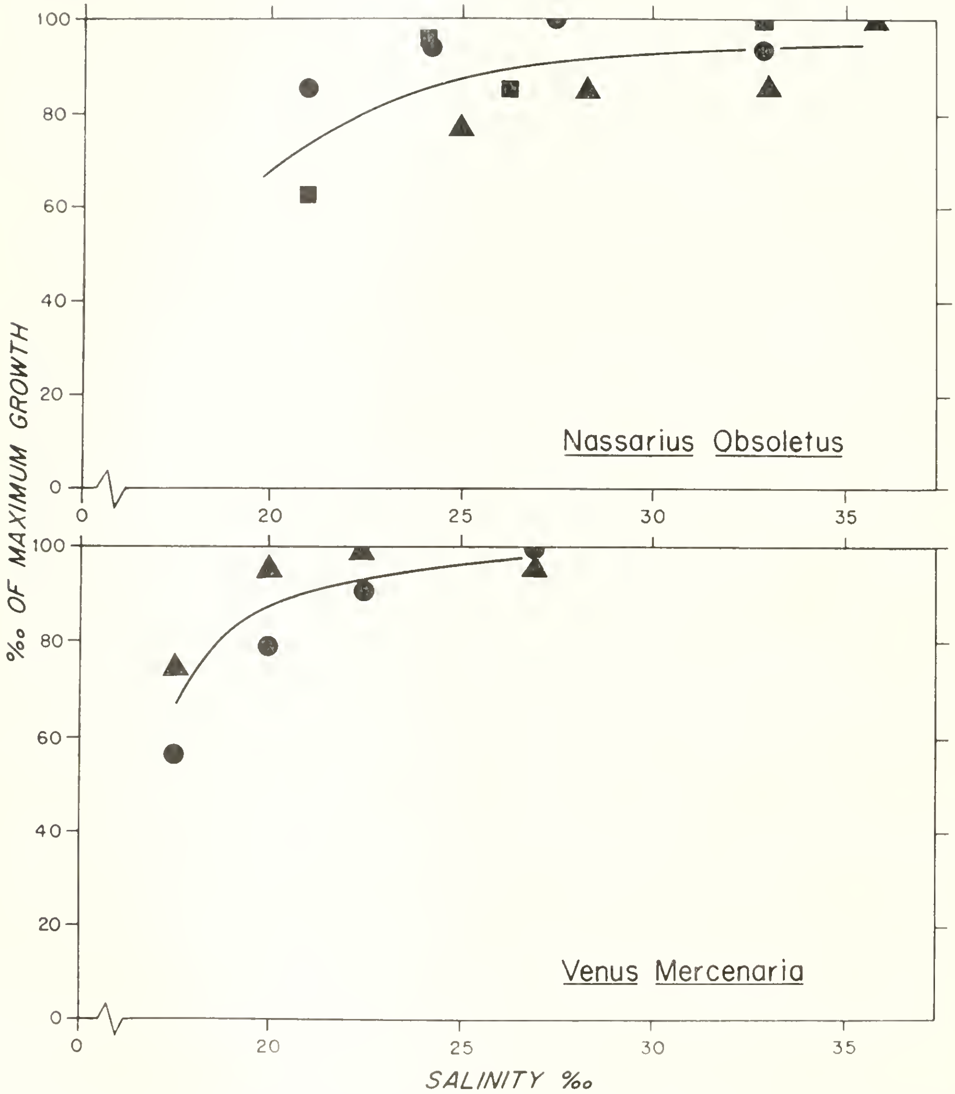

FIGURE 5. Percentage growth obtained at various reduced salinities relative to the maximum growth obtained at optimal salinity conditions in the gastropod, *Nassarius obsoletus* Say and pelecypod, *Venus mercenaria* L. Data for *V. mercenaria* are from Davis (1958), p. 301, Fig. 1. Growth data for each species were used only if more than 5% of the larvae in the culture completed development to metamorphosis. The data refer to 15 days after the beginning of planktotrophic life in *N. obsoletus* (*i.e.*, creeping-swimming stage) and 12 days after fertilization in *V. mercenaria*. The different conventions indicate values for individual series of experiments. The curves are only intended to be suggestive.

TABLE IV

Maximum differences of larval growth rate attributable to various ecological factors
(Average values of I from experimental data)

	N. obsoletus	*V. mercenaria*
Physical characteristics		
Temperature		
Range within which development is completed	17.5° to 30° C.*	18° to 30° C.**
Average difference in growth rate between optimum and minimum required for complete development	50%*	60%**
Salinity		
Range within which development is completed	>17.7‰	>15.0‰***
Average difference in growth rate between optimum and minimum required for complete development	20%	34%***
Extreme difference in growth rate observed between optimum and minimum required for complete development	13 to 40%	22 to 45%***
Biological characteristics		
Concentration of algal food (Differences between optimum and minimal growth obtained at concentrations between 2.5×10^{-3} and 40×10^{-3} mm.^{3} packed cells per 3-l. culture)		
Isochrysis galbana	—	17.7%****
Monochrysis lutheri	—	51.2%****
Chlorella sp. (causes inhibition of growth and death of larvae at highest concentrations)	—	29.7%****†
Phaeodactylum tricornutum (data from relative concentrations only)	*ca.* 50% (preliminary estimation)	—
Species of algal food (based on 10 species of or combination of species when equal packed cell volumes were used)	—	Up to 75%††

* Scheltema (1963), p. 17, Fig. 2.
** Loosanoff *et al.* (1951), p. 71, Table III; Loosanoff (1959), p. 315, Fig. o.
*** Davis (1958), p. 301, Fig. 1.
**** Davis and Guillard (1958), p. 302, Fig. 6.
† This figure represents difference in growth at concentrations between 2.5×10^{-3} and 20×10^{-3} mm.^{3} per 3-liter culture. At higher concentrations larvae did not survive.
†† Davis and Guillard (1958), p. 298, Fig. 3; p. 299, Fig. 4.

Because differences in the nutritional value of the algal food cells used in growth experiments are not readily controlled (Walne, 1963), it is not possible to compare the results from one series of experiments directly with the next without elaborate experimental procedures. A direct comparison between most series of growth experiments usually shows large discrepancies. Only cultures of larvae simultaneously grown using the same source of algal food can be directly compared with one another. It is possible, however, to compare differences in computed growth rates. Likewise the per cent inhibition, *I*, computed from the equation given above, can be directly compared if the differences in length are derived from samples of similar size. Using this kind of information it is also possible to compare the relative importance of reduced salinity to growth between species of mollusks. In Figure 5 this has been done for two species which show similar distributions within estuaries along the Atlantic coast of the United States: the data of Davis (1958) on the growth of larvae of the pelecypod, *Venus mercenaria,* are compared with growth data from the larvae of *N. obsoletus.* The conclusion may be made that salinity little affects larval growth in either species until the lower limit of salinity tolerance is approached. Only the lower third of the salinity range has any marked inhibitory effect on growth and there is no simple linear relationship between growth and the salinity level. The similarity in response of the two species, *V. mercenaria* and *N. obsoletus,* is striking.

The importance of salinity relative to other factors affecting larval growth

An indication of the relative importance of salinity to larval growth can be had by comparing its maximum effect relative to that of some other factors known to control growth rate of *N. obsoletus* and *V. mercenaria.* By tabulating the values for maximum percentage difference obtained from that of optimum growth, the importance of various ecological factors in limiting growth rate becomes apparent. This is shown in Table IV. Here the range within which completion of development occurs is given for physical characteristics of the environment, and the percentage values are average maximum differences in growth attributable to these physical factors. Differences in growth rate under different biological conditions for which data are available, *viz.* concentration and species of algal food, are given within the limits of concentrations indicated. The maximum inhibition of growth varies with the algal species. On the basis of the figures given, the concentration and the species of algal food usually affect growth rate of *V. mercenaria* much more than any of the physical factors of the environment. There is preliminary evidence that this is also true for *N. obsoletus* larvae. The food value of algal cells to mollusk larvae in relation to the conditions under which the algal cells were grown is not yet known (see Walne, 1963). The table shows that the importance of low salinity in inhibiting growth rate, within the limits in which development of the larvae is completed, is certainly minimal, less than any other factor in the environment known to retard growth of the larvae.

This work is dedicated to the memory of G. Francis Beaven, who in his quiet way first interested me in the relationship between salinity and the distribution and survival of estuarine organisms.

SUMMARY

1. There is no large difference between the lower lethal salinity for the veliger larva and the adult of *Nassarius obsoletus.* The region of stress in snails is between 12.5‰ and 13.5‰; that of the early larva is between 14.0‰ and 15.5‰. Throughout larval development no change occurred in the value of the lower lethal salinity.

2. Difference in growth rate of *N. obsoletus* larvae observed at salinities above 24‰ are usually slight. However, at 21‰ and less there is a statistically sig-

nificant drop in growth rate, while at a mean salinity of 17.7‰, it was not possible to rear the larvae to the completion of development and metamorphosis. The maximum inhibition of growth attributable to the affects of salinity, within the range at which development of the larvae is completed, is between approximately 13% and 40% and averages about 20%. This is less than that of other ecological factors known to retard growth.

3. The net result of reduced salinity, within the lower third of the range at which the larval development of *N. obsoletus* is completed, is an increase in the length of time to reach the creeping-swimming stage which precedes metamorphosis, and an increased mortality of larvae as the limit of salinity tolerance is reached.

4. A comparison of data from *N. obsoletus* with that of another molluscan species, the pelecypod *Venus mercenaria,* which is found at approximately the same salinities within Atlantic coast estuaries along the United States, shows striking similarities.

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PHYSIOLOGICAL SALT SOLUTION FOR THE LAND CRAB, GECARCINUS LATERALIS

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The land crab, *Gecarcinus lateralis,* is an active responsive animal until it approaches ecdysis; in the few weeks before and after ecdysis, however, the animal becomes lethargic (Bliss, 1962). These variations in activity may well be related to the marked changes in the metabolism of somatic muscle during molting (Skinner, 1962; 1963a, 1963b). In order to investigate the physiology of this muscle it has first been necessary to develop a Ringer's solution appropriate to the animal. This paper describes analyses performed on *Gecarcinus* serum. Observations on osmoregulation are included. From these data a Ringer's solution has been devised and tested.

MATERIALS AND METHODS

1. *Animals*

Some specimens of *Gecarcinus lateralis* were collected in the field at Bimini and were used immediately at the Lerner Marine Laboratory. Other specimens were shipped from the Bermuda Biological Station to New York and were housed in covered aquaria containing sand moistened with tap water. A fingerbowl of sea water was available in each tank; where indicated, tap water was substituted for sea water.

2. *Preparation of blood serum*

Blood was collected from the cut appendage of an animal which had been acutely chilled to prevent autotomy of the cut limb. The clot was mechanically disrupted and sedimented by centrifugation.

3. *Osmolality, sodium, potassium and chloride*

Immediately after preparation, the osmolality of the serum was measured in a Fiske osmometer. Concentrations of sodium and potassium in the serum were determined by standard flame-photometric methods, using LiCl as an internal standard.

Chloride was determined by the Cotlove titrimetric method (Cotlove *et al.*, 1958). Initial attempts to determine chloride concentration of untreated blood serum led to results varying by as much as 15% on five replicate samples. Since the protein concentration of *Gecarcinus* serum is high and variable (2% to 10%, unpublished data), we thought that protein might be interfering with the analyses.

Consequently, the protein precipitated by the nitric-acetic acid reagent used in the analysis was homogenized to free any trapped chloride and removed by centrifugation. Aliquots of the supernatant were used for the titration. This procedure reduced the variability between replicate samples to less than 2%.

4. *Calcium and magnesium*

(a) *Preparation and characterization of an ultrafiltrate.* Blood was ultrafiltered to obtain a value for free calcium and magnesium without including divalent ions associated with proteins. Three-inch dialyzer tubing (average pore diameter 48 Å) was cut along its edge, giving a piece 6 inches wide. This was shaped into a sack and inserted into the top of a 12-ml. conical centrifuge tube. One to 2 ml. of blood were introduced into the sack which was then tightly stoppered and centrifuged. The first fluid collected after bringing the centrifuge to speed was set aside as possible condensate from the tubing. TCA was added to each of these initial collections. In the rare event that any precipitate formed (indicating the presence of protein and hence a leak in the system), the sample was transferred to another dialysis sack. The tubes were then spun at 3000 rpm in a model CM International centrifuge for two hours. Heating was prevented by packing the drive shaft of the centrifuge in dry ice during the centrifugation. The rate of ultrafiltration was about 0.025 ml. hr.^{-1} ml. serum^{-1}. The ultrafiltrate obtained was colorless and contained no more than .06% protein (as determined by the method of Lowry *et al.*, 1951), representing about 1% of the protein initially present in the serum. Within the limits of experimental error, the alkali metal concentrations in the ultrafiltrate were the same as those in whole serum. Since the small correction for serum water would be opposite to that applied for the Donnan equilibrium, the similarity was an expected result, and indicated that there was no significant evaporation of the ultrafiltrate during preparation.

(b) *Assay method.* The dye Eriochrome Black T is pink when chelated to divalent cations and blue when free in solution after the cations have been removed by a stronger chelating agent. With this dye as an indicator, the sum of calcium and magnesium was titrated with EDTA at a basic pH in the presence of cyanide (Ames and Nesbett, 1958). Calcium alone was determined titrimetrically on separate aliquots of each sample, using 2-hydroxy-1-(2-hydroxy-4-sulfo-1-naphthyl-azo)-3-naphthoric acid (HHSNN, Fisher Scientific Company) as indicator and EGTA (ethylene glycol bis (β-aminoethyl ether)-N, N-tetraacetic acid) as the titrant (Weber and Herz, 1963). Magnesium was obtained by subtracting the calcium value from the total. Standard curves were run with each set of experimental samples.

5. *Sulfate*

Proteins were precipitated from serum with perchloric acid (0.7 *M* final concentration). The supernatant was neutralized with KOH and the concentration of inorganic sulfate measured according to the method of Jones and Letham (1956). In four experiments, where known amounts of sulfate were added to crab serum, 101.5% of the added sulfate was recovered.

6. *pH, pCO_2, pO_2, and bicarbonate*

Blood was collected by immersing a cut appendage below the surface of paraffin oil saturated with water. The clot was mechanically disrupted and the serum transferred anaerobically to a cuvette housing a Clark polarographic O_2 electrode, a Severinghaus CO_2 electrode, and a glass pH electrode, all of which were read out by means of a Beckman model 160 gas analyzer. The pH of any sample which differed significantly from the others was measured independently with a Radiometer pH meter; in such cases the two readings always checked within 0.02 pH unit. In addition pH measurements were made after equilibration of samples with varying concentrations of CO_2 in air, in order to obtain the apparent pK. With this pK and the measured pH and pCO_2, the bicarbonate concentrations were calculated.

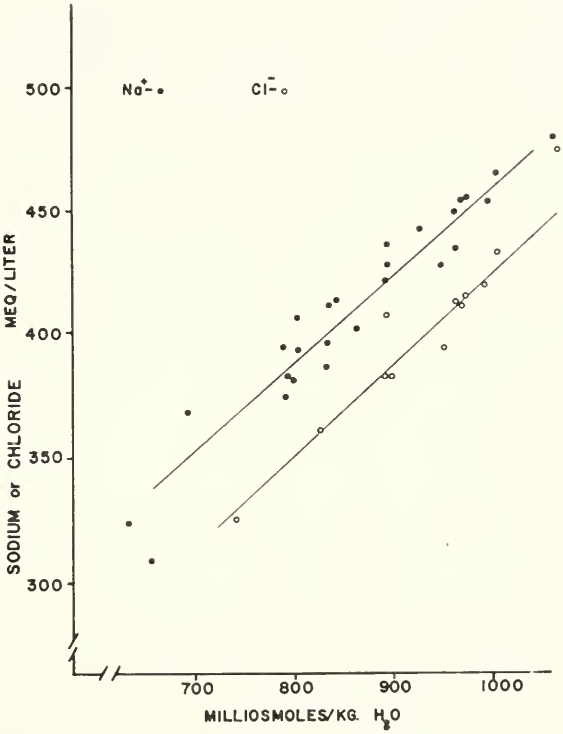

FIGURE 1. Sodium and chloride as functions of osmolality in the serum of *Gecarcinus lateralis*. *Sodium data:* closed circles; *chloride data:* open circles. Regression lines were fitted to the data by the method of least squares.

7. *Inorganic phosphate*

Protein was precipitated from serum with TCA (trichloroacetic acid) at a final concentration of 5%. Inorganic phosphate was determined by the method of Fiske and SubbaRow (1925).

RESULTS AND DISCUSSION

1. *Osmolality, sodium, potassium and chloride*

The osmolality of *Gecarcinus* serum varied from 610 to 1060 mosm/kg. H_2O, depending on environmental conditions. Sodium varied from 310 to 480 meq/L. and, in any given animal, accounted for approximately one-half the total osmolality (Fig. 1). Chloride, the principal serum anion, was also linearly related to the osmolality but was present at concentrations about 35 meq/L. less than the sodium in the 12 sera analyzed for both ions.

Serum potassium varied from 7 to 15 meq/L. Figure 2 shows that the potassium concentration also tended to vary with osmolality, but in this case the data were proportionately more scattered and the interdependence was not as evident until we obtained the data on osmoregulation described below.

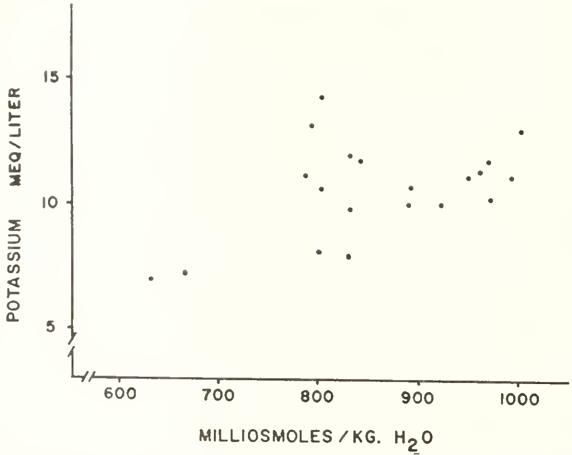

FIGURE 2. Potassium as a function of osmolality in the serum of *Gecarcinus lateralis*.

During the initial phases of this work at Bimini, 21 animals sampled immediately after they were caught in the field had an average serum sodium of 369 ± 28 (S.D.) meq/L., while 8 animals kept on moistened sand (but with no other source of water) for 48 hours before sampling had an average serum sodium of 456 ± 26.3 (S.D.) meq/L. This difference was highly significant and prompted further investigation of the effects of environmental conditions.

In Bimini, *Gecarcinus* burrows in the sand some distance from the sea in an area where the ground water is salty. Animals shipped to New York have, shortly after arrival, a serum osmolality of approximately 830 mosm/kg. H_2O. A group of animals was kept from arrival in an aquarium with sea water[1] available in the water bowls; after several weeks a blood sample was taken for osmolality, sodium, potassium and chloride determinations. The animals were replaced in tanks with tap water in the bowls, and after 8 days (three animals) or 27 days (two animals), the blood collections and determinations were repeated. The results (Table I) show that all four parameters decreased when only fresh water was available, and that the decrease was greater after the longer exposure. These experiments show that potassium, as well as sodium and chloride, does vary in the same sense as the osmolality, a relationship not readily seen from the data in Figure 2.

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[1] The sea water used was obtained from the New York Aquarium (Coney Island) and had the following measured composition (in meq/L.): Sodium, 375; potassium, 8.1; chloride, 460; magnesium, 79.0; calcium, 16.4; osmolality = 860 mosm/kg. H_2O; salinity = 27.1‰.

2. *Calcium and magnesium*

The mean value for free calcium in serum water of intermolt animals was 17.2 ± 2.4 (S.D.) meq/L.; the mean value for free magnesium was 13.8 ± 2.2 (S.D.) meq/L. (Table II).

TABLE I

Osmolality, sodium, potassium and chloride of Gecarcinus serum in animals given access to sea water only for several weeks (I) and thereafter given access to tap water only (II)

Animal	Days of access to tap water	Osmolality (mosm/kg. H_2O)		Sodium (meq./L.)	Chloride (meq./L.)	Potassium (meq./L.)
1	8	I	1000	467	435	13.0
		II	890	423	385	10.8
2	8	I	965	456	414	11.7
		II	890	438	410	10.5
3	8	I	990	456	422	11.1
		II	960	452	415	11.3
4	27	I	948	430	396	11.2
		II	825	388	362	8.0
5	27	I	1058	457	418	11.5
		II	895	430	385	10.0

These values are distinctly lower than those found by Gross (1963) for *Gecarcinus.* The difference probably reflects the fact that Gross dialyzed the serum against distilled water (for his method, see Gross, 1959), a procedure which would be expected to release cations normally bound to protein.

We found that two premolt animals had calcium concentrations higher than the average intermolt level, whereas the magnesium levels were the same at both stages (Table II). After ecdysis, the calcium level fell while the magnesium level rose more than 40%. Travis (1955) has described a similar pattern for calcium in the pre- and postmolt periods for another crustacean, the spiny lobster. Although these changes are of interest in the overall electrolyte metabolism of molting, we considered them too small to influence significantly the physiological effectiveness of a Ringer's solution; therefore we did not sample a larger series of pre- and postmolt animals.

TABLE II

Concentration of calcium and magnesium in Gecarcinus serum ultrafiltrate

Stage	Animal	Calcium (meq./L.)	Magnesium (meq./L.)
Intermolt	1	13.2	15.2
	2	18.5	11.5
	3	17.4	15.7
	4	24.8	10.0
	5	18.4	17.6
	6	17.1	15.3
	7	19.2	13.2
	8	17.6	12.8
	9	16.8	12.4
	10	16.8	14.4
	11	14.3	
	12	13.5	Avg: 13.8 ± 2.2 (S.D.)
		Avg: 17.2 ± 2.4 (S.D.)	
Premolt	1	22.0	14.0
	2	24.8	13.6
		Avg: 23.4	Avg: 13.8
Postmolt	1	18.4	17.6
	2	20.0	18.0
	3	18.8	23.2
		Avg: 19.1	Avg: 19.6

3. *Inorganic sulfate*

The results of 17 analyses are listed in Table III. Sera from 13 intermolt animals had an inorganic sulfate concentration of 11.18 ± 0.66 (S.D.) meq/L. Two premolt and two postmolt animals had similar values, indicating no variation during the molt cycle.

4. *pH, pCO_2, pO_2 and bicarbonate*

Gecarcinus blood serum has a relatively constant pH of 7.2 and a pCO_2 of 14 mm. Hg (Table IV). The wide fluctuations observed in the oxygen tension are unexplained. They are probably not due to the mixing of "arterial" with "venous"

TABLE III

Inorganic sulfate in Gecarcinus serum

Stage	Number of animals	Inorganic sulfate	
		Range (meq./L.)	Average (meq./L.)
Intermolt	13	10.20–11.94	11.18 ± 0.66 (S.D.)
Premolt	2	10.86–11.06	10.96
Postmolt	2	10.92–11.68	11.30

TABLE IV

pH, pCO_2, pO_2, bicarbonate in Gecarcinus serum

Animal	pH	pCO_2 (mm. Hg)	pO_2 (mm. Hg)	Bicarbonate (meq./L.)
1	7.20	14	24	7.40
2	7.22	13	29	5.14
3	7.43	12	28	7.70
4	7.14	12	72	3.95
5	7.05	16	56	4.15
6	7.08	15	36	4.99
7	7.26	16	30	6.94
8	6.95	14	46	2.97
Averages	7.17	14	40	5.40

blood (if such terms can be used to describe the hemolymph of an arthropod), since if mixing were the cause of the variability, we would expect low pO_2 values to be correlated with high pCO_2 values.

The pCO_2 is considerably higher than that of the sera of many other invertebrates (Spector, 1956; p. 270). The low pCO_2 of insects is probably due to the direct oxygenation of every cell by tracheole penetration, while the low pCO_2 of various marine Crustacea is probably due to the solubility of CO_2 in the sea water bathing the gills.

To determine the site of the diffusion barrier for CO_2 in *Gecarcinus,* the branchial chamber of an animal was flushed with 100% O_2 for 10 minutes before and throughout the collection of the blood sample. The pO_2 of that serum was only 52 mm. Hg, while the pCO_2 was 13.5 mm. Hg. The maintenance of this high pCO_2 in the blood despite the fact that the gill chamber was flushed free of CO_2 indicates that the barrier lies between the gill chamber and the branchial chamber. Further experiments will be performed to test this possibility.

5. *Inorganic phosphate*

The inorganic phosphate content of 31 serum samples collected from animals in the field averaged 0.76 but ranged from 0.21 to as high as 2.08 mmols/liter (Table V).

TABLE V

Inorganic phosphate in Gecarcinus serum

Stage	Conditions	Number of animals	Inorganic phosphate range (mmoles/L.)	Average
Intermolt	Collected in field	31	0.21–2.08	0.76
Intermolt	Starved >3 days	7	0.29–0.53	0.42
Premolt ($D_0 - D_4$)	Did not eat after onset of D_0	8	0.33–0.78	0.50
Day of ecdysis		1	0.42	0.42
Postmolt ($A - C_1$)		12	0.34–0.66	0.51

Travis (1955) found that under controlled feeding conditions the level of inorganic phosphate in the serum of the spiny lobster remained relatively constant throughout the molt cycle. A decrease of 25% in the postmolt period was the greatest fluctuation she observed. According to Travis, diet was the principal factor which determined serum phosphate levels.

We took our blood samples in the field within a few hours after the animals had been collected; therefore, the time and content of each specimen's most recent meal probably accounted for the 10-fold variation in inorganic phosphate level. More recently we have analyzed blood from a group of animals maintained in the laboratory, where feeding conditions could be controlled. Sera from these animals starved for three or more days showed much less variation in the inorganic phosphate concentration and were in the lower range of those collected in the field (Table V).

There was no correlation of inorganic phosphate concentration with the molting cycle. These data appear to be similar to those obtained by Travis for the spiny lobster.

TABLE VI

Composition of Ringer's solution for Gecarcinus

Compound	mmols./L.
NaCl	430
K_2SO_4	5
$MgCl_2$	7
$CaCl_2$	9
"Tris" buffer	10

The final pH is adjusted to 7.2 with 0.2 N maleic acid.

6. *Preparation and physiological efficacy of the Ringer's solution*

Based on the measurements above a Ringer's solution has been devised (Table VI).[2] Regardless of the serum osmolalities (and corresponding ion concentrations) within the range of 610 to 1060 mosm/kg. H_2O, there were no gross behavioral differences in the specimens of *Gecarcinus*. Hence it appeared that a Ringer's solution within this range should support normal neuromuscular activities. We have selected a sodium concentration (430 meq/L.) and osmolality close to the values observed in animals shortly after their arrival in the laboratory. The use of chloride salts raised the concentration of chloride somewhat higher than any observed in the animals. Since the final Ringer supported prolonged neuromuscular activity, the high chloride does not appear to exert any deleterious effect.

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[2] Previous data published on the ionic composition of *Gecarcinus lateralis* serum by Prosser and Brown (1962; p. 60) were preliminary data obtained by J. W. Green in collaboration with one of us (DMS). Since for technical reasons we were not confident of the validity of some of the numbers obtained at the time, we did not publish the data. However, we did make them available to a few colleagues, one of whom submitted them for publication to Dr. Prosser.

Dr. Prosser published them in good faith without knowledge of their source. Before undertaking the present work, we tested a Ringer's solution prepared from the values obtained earlier and found that it did not support nerve or muscle function.

The solution is brought to pH 7.2 with Tris(hydroxymethyl) aminomethane/maleic acid, a buffer commonly used in crustacean Ringer's solution (Elliott and Florey, 1956). The concentration of inorganic phosphate in *Gecarcinus* plasma was too low to use it as an effective buffer. Indeed, the small concentration prompted us to omit inorganic phosphate from the solution entirely. We have also omitted bicarbonate from the Ringer since its buffering capacity at 5×10^{-3} M would be small and would require in any case the maintenance of a constant pCO_2.

The measured osmolality of the final solution was 850 mosm/kg. H_2O.

A chela of an intermolt animal was removed and the nerve trunk in the merus was freed of all surrounding tissue. Forty ml. of the Ringer were perfused through the cut end of the propus to wash out blood. The nerve trunk was stimulated and the contraction of the adductor muscles in the propus was observed intermittently over a four-hour period. During the same period of time, sensory stimulation (*i.e.*, light taps) in the region of the mechanoreceptors in the leg joints elicited action potentials which could be recorded approximately 4 cm. down the sensory nerve. Thus, axonal conduction, neuromuscular transmission, and muscular contraction appear normal for up to four hours, at which time the experiments were terminated.

Conclusions

Summing the inorganic ions for an animal with an osmolality in the median range, *e.g.*, 850 mosm/kg. H_2O, we find that the inorganic cations total about 450 meq/L. and the inorganic anions about 385 meq/L., leaving 65 meq/L. anionic charge unaccounted for. Acidic amino acids contribute little to this charge since they are present in low concentrations (*ca.* 0.05–0.10 mmol/liter total) and are more than balanced by basic amino acids (*ca.* 0.13–0.74 mmol/liter, unpublished data). The protein concentration in *Gecarcinus* serum is high and the isoelectric points of all the proteins are not known. Most should be negatively charged at the pH of the animal's plasma unless the isoelectric points are unusually basic. Therefore negative charges on protein probably account for many of the undetermined anions.

In a serum of osmolality 850 mosm/kg. H_2O the total of all inorganic ions is about 815 mmol/L. If we assume a rational osmotic coefficient of about 0.9 for these electrolytes, only 13% of the total osmotic pressure is unaccounted for. Much of this will be made up by proteins, amino acids, glucose, and other commonly occurring organic solutes. Therefore it is unlikely that any single organic compound makes up an important fraction of the total osmotic pressure, as does urea in elasmobranchs (Prosser and Brown, 1962; p. 142) and glycerol in some insects (Wyatt and Meyer, 1959).

The clear dependence of sodium, chloride and potassium concentrations and of osmolality on the nature of the available water supply indicates that *Gecarcinus* does not regulate these concentrations about a critical set point. A corollary of this conclusion is the observation that all animals showed similar motor behavior regardless of their plasma ion concentrations. It is of interest that the serum osmolality is always greater than that of the available water supply. *Gecarcinus* is a land animal and evaporation at its gills undoubtedly leads to the observed hemoconcentration. Gross' data (1963) show that concentrations of alkali metal cations are essentially the same in urine as in blood over a wide range of blood concentrations. This observation precludes the possibility that renal mechanisms compensate blood changes.

Gross measured sodium and potassium in groups of *Gecarcinus* exposed to a variety of environments; he concluded, as have we, that the concentrations of these two ions are not closely regulated. Flemister (1958) immersed the animals in aqueous solutions of various chloride concentrations and found that even after several days the blood chloride did not equal environmental chloride. Under these conditions Flemister noted that animals immersed in hypotonic sea water had blood chloride concentrations greater than that of the environment, whereas animals immersed in hypertonic sea water had blood chloride less than that of the environment. In his experimental situation the normal evaporative processes are prevented. However, his data indicate, as do ours, that the plasma chloride concentration decreases in a hypotonic medium and increases in a hypertonic environment.

Whether one concludes that *Gecarcinus* is capable of osmoregulation depends to some extent on one's definition of the term. Serum ion and osmolality levels are not maintained constant independent of the environment; but not even in the case of total immersion do they equilibrate with the environment. In their normal terrestrial habitat evaporative losses can and do occur, and the animals appear to compensate for these in the laboratory by spending some time in the available water supply (personal observations; see also Gross, 1963). Thus we may conclude that the animals are capable of osmoregulation only to a limited extent (in part by behavioral mechanisms), and that the resulting fluctuations in serum concentrations are readily tolerated.

We wish to express our appreciation to the staff of the Lerner Marine Laboratory, Bimini, where this work was initiated; to Arnold Davidson for excellent technical assistance; to Dr. E. Bergofsky for performing some of the analyses and Dr. M. Mendelson for performing some of the tests on the efficacy of the Ringer. This work was supported by USPHS grant #AM 06268 to one of us (DMS) and by ONR assistance which made the preliminary work in the field possible.

SUMMARY

1. From determinations of the principal electrolytes and respiratory gases in the serum of the land crab, *Gecarcinus lateralis,* a Ringer's solution has been devised and found to be effective in supporting neuromuscular activity for at least four hours in isolated preparations.

2. The animal is capable of a limited osmoregulation.

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CHLOROPLAST PIGMENTS AND THE CLASSIFICATION OF SOME SIPHONALEAN GREEN ALGAE OF AUSTRALIA[1]

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With respect to their chloroplast pigments, the siphonaceous green algae of the order Siphonales (class, Chlorophyceae) differ slightly from the common unicellular and multicellular green algae (Strain, 1958, pp. 37, 162, 167). From this standpoint of pigment composition, the Siphonales are not so closely related to the other green algae as all the other uninucleate and multinucleate Chlorophyceae are related to one another (Fritsch, 1935, pp. 60, 368; Smith, 1955, p. 101).

From the nature and the proportions of their chloroplast pigments, the Siphonales are remotely related to the other siphoneous or coenocytic algae such as the Vaucheriaceae (Strain, 1958, pp. 51, 169), which are alternatively classified as Siphonales (Fritsch, 1935, p. 368) or as Heterosiphonales (class, Xanthophyceae or Heterokontae) (Smith, 1955, p. 177; Taylor, 1960, p. 190). With respect to their pigments, the Heterosiphonales are similar to a group of multicellular filamentous algae known as the Heterotrichales (Tribonemataceae), also of the class Heterokontae (Smith, 1955, p. 174; Strain, 1951, p. 254, 1958, p. 169). This relationship, based on pigments, supports the contention that the Heterosiphonales (Vaucheriaceae) and the Heterotrichales (Monociliaceae and Tribonemataceae) belong to the same class (Heterokontae) (Smith, 1955, p. 166). With respect to pigments, *Dichotomosiphon tuberosus* (A.Br.) Ernst, a fresh-water, siphonaceous species, is related to the Siphonales (Strain, 1958, pp. 37, 167) (predominantly marine species), not to the siphonaceous Heterosiphonales (predominantly fresh-water species) (Fritsch, 1935, p. 369; Smith, 1955, pp. 101, 115; Taylor, 1960, p. 190).

Like all the common green algae, and the higher plants as well, the species of the Siphonales examined thus far contained chlorophyll *a* plus chlorophyll *b*. The heterosiphonalean and heterotrichalean species contained chlorophyll *a* unaccompanied by other chlorophylls (Strain, 1958, pp. 37, 51, 167, 169). Like the common green algae, the coenocytic species of the order Siphonales contained the principal xanthophylls neoxanthin, violaxanthin and lutein plus traces of zeaxanthin. They also contained an additional xanthophyll, called siphonaxanthin, and an ester of this pigment called siphonein. The heterosiphonalean and the heterotrichalean species contained a group of unnamed xanthophylls not observed elsewhere (Allen *et al.*, 1964; Strain, 1958, p. 51). The green algae of the order Siphonales contained much more α-carotene than β-carotene. The algae of the heterokontaen groups contained principally β-carotene. *Dichotomosiphon* contained the same chlorophylls and xanthophylls found in the Siphonales, but the principal carotene was the β-isomer (Strain, 1958, p. 167).

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[1] Based on work performed under the auspices of the U. S. Atomic Energy Commission and the Australian Academy of Science.

The siphonalean green algae available for the earlier studies (Strain, 1958, p. 167) included about 24 varieties and species. They represented 9 genera and 5 families. Except for *Dichotomosiphon*, obtained from Lake Michigan, these species were indigenous to the coastal waters of California, Hawaii and Puerto Rico.

Materials and Observations

As an extension of the earlier studies, the pigments of several Australian species of siphonaceous, green algae have now been isolated and compared with the pigments described before (Strain, 1958). The organisms were collected at various remote locations in the Southern Hemisphere (Womersley, 1959) at low tide during the spring months, August through November, 1963. They were examined soon after collection. The pigments were separated and compared by chromatography in columns of powdered sugar with petroleum ether plus 0.5% *n*-propanol as the wash liquid (Strain, 1958, p. 27). In the United States the powdered sugar is sold as Confectioners Powdered Sugar. In Australia it is available as Soft Icing Mixture and as Icing Sugar. The carotenes were separated in columns of activated magnesia [Micron Brand magnesium oxide No. 2641 (Strain, 1958, p. 30) also sold as Sea Sorb 43 by Fisher Scientific Company]. Before the columns were packed, the magnesia was mixed with twice its weight of heat-treated siliceous earth (Celite 545).

The individual pigments with their sequence (top to bottom) and color in the columns of powdered sugar were:

Siphonaxanthin, red-orange
Neoxanthin, light yellow
Violaxanthin, light yellow
Siphonein, red-orange
Chlorophyll *b*, yellow-green
Lutein ± zeaxanthin, yellow
Chlorophyll *a*, green
α-Carotene + β-carotene, yellow

The location of siphonein was previously reported below the chlorophyll *b* due to mislabelling of the chlorophyll *b* and siphonein zones (Strain, 1958, p. 38). Separate chromatographic experiments also revealed that siphonein and fucoxanthin, the principal xanthophyll of diatoms and brown algae, are adsorbed together in the sugar columns. Both pigments form zones of nearly the same color above the chlorophyll *b* zone. When adsorbed together in sugar columns, these two xanthophylls are incompletely separated, even after prolonged washing with the petroleum ether-propanol solvent. Reaction of the fucoxanthin, in ether solution, with concentrated hydrochloric acid, to yield a blue color in the acid layer, serves to distinguish the fucoxanthin from the siphonein, which does not yield a blue color.

The siphonalean algae that were examined are listed in Table I along with the regions where they were collected.

All the species included in Table I yielded much more α-carotene than β-carotene. All these species except *Caulerpa filiformis* contained siphonaxanthin and siphonein in addition to the other chloroplast pigments found in green algae and higher plants (Strain, 1958, pp. 136, 157, 162). They yielded these pigments in the chromatographic sequence indicated above. *Chlorodesmis comosa* yielded very little of the siphonaxanthin and siphonein.

Because of the absence of siphonaxanthin and siphonein in *Caulerpa filiformis,* analyses were repeated several times. The organism was also collected from two different stands on neighboring rocks, but the siphonaxanthin and siphonein, found in other species of *Caulerpa* as well as in all the other species of the Siphonales, could not be detected.

TABLE I

Algae of the order Siphonales, whose chloroplast pigments were determined, and the locations where they were collected in Australia

Bryopsidaceae	
Bryopsis sp.	Cottesloe Beach (Perth)
Caulerpaceae	
Caulerpa cupressoides (West) C.Ag.	Heron Id. (Gt. Barrier Reef)
Caulerpa distichophylla Sonder	Cottesloe Beach (Perth)
Caulerpa filiformis (Harv.) C.Ag.	Cronulla Beach (Sydney)
Caulerpa lentillifera J. Ag.	Redcliff (Brisbane)
Caulerpa racemosa (Forssk.) J.Ag.	Heron Id. (Gt. Barrier Reef)
Caulerpa racemosa var. *laetevirens* (Mont.) Weber-van Bosse	Pt. Peron (Freemantle)
Halimeda discoidea Decaisne	Heron Id. (Gt. Barrier Reef)
Codiaceae	
Chlorodesmis comosa Harv. & Bail.	Heron Id. (Gt. Barrier Reef)
Codium duthieae Silva	Pt. Peron (Freemantle)
Codium fragile (Suring) Hariot	Hungry Pt. Cronulla (Sydney)
Codium lucasii Setch.	Cronulla (Sydney)
Codium lucasii Setch.	Redcliff (Brisbane)
Codium spongiosum Harv.	Heron Id. (Gt. Barrier Reef)

Cladophoropsis herpestica (Mont.) Howe (Valoniaceae), collected at Redcliff (Brisbane), yielded the pigments characteristic of the uninucleate green algae. Siphonaxanthin and siphonein were absent, and β-carotene was the principal carotene isomer. These results conform to those obtained with other species of the Valoniaceae, Boodleaceae and Anadyomenaceae (order, Siphonocladales) (Strain, 1958, p. 164).

CONCLUSIONS

On the basis of the species available thus far, siphonaxanthin and siphonein are constituents of siphonalean green algae indigenous to remote regions both of the Northern and Southern Hemispheres. The absence of these two pigments in *Caulerpa filiformis* indicates that siphonaxanthin and siphonein are not definitive characters for the classification of all the Siphonales. The preponderance of α-carotene relative to β-carotene is without exception in the marine Siphonales. The fresh-water Dichotomosiphon is an exception (Strain, 1958, p. 157).

The wide distribution of siphonaxanthin and siphonein in the species examined (Table I) indicates that the Siphonales comprise a distinct group. This conclusion is supported by the preponderance of α-carotene over β-carotene in all the marine species.

The absence of siphonaxanthin and siphonein and the preponderance of β-carotene in *Cladophoropsis herpestica* agree with earlier analyses of other species of the Siphonocladales (Strain, 1958, p. 164). This result indicates that the Siphonales stand apart from the Siphonocladales.

The universal occurrence of chlorophyll *b* along with chlorophyll *a* supports the view that the Siphonales and the Siphonocladales are major divisions of the Chlorophyceae. This relationship is supported by the occurrence of the same xanthophylls, namely, lutein, violaxanthin and neoxanthin, in the Siphonales, in the Siphonocladales and in all the other species of the Chlorophyceae.

There appears to be no relationship among the occurrence of particular chloroplast pigments, the calcification of the organisms and the composition of the structural polysaccharide in the marine Siphonales (Feldmann, 1946; Fritsch, 1935, p. 368; Miwa *et al.,* 1960; Smith, 1955, p. 12).

The occurrence of chlorophyll *a* and β-carotene in the *Siphonales* and in the *Heterosiphonales* indicates a basic relationship between these two groups. This relationship is akin to that existing among all autotrophic organisms in which oxygen production is always associated with the presence of chlorophyll *a* and α- or β-carotene (Strain, 1951, 1958, p. 83, 1964).

In conjunction with the earlier pigment studies (Strain, 1958), the current results support the classification of the Heterosiphonales with the Heterotrichales in a class apart from the Chlorophyceae.

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Summary

1. The chloroplast pigments of twelve species of siphonalean green algae native to the marine waters of Australia were isolated by chromatography.

2. All these siphonalean species but one contained the same pigments found in the siphonalean Chlorophyceae of the Northern Hemisphere.

3. *Caulerpa filiformis* lacked the siphonaxanthin and siphonein associated with the chlorophylls and carotenoids in the other Siphonales, but it contained a preponderance of α-carotene relative to β-carotene as did all the other Siphonales.

4. These observations indicate that the Siphonales are significantly different from the other green algae, but they are more closely related to the Chlorophyceae than to any other algal group.

5. The pigment distribution supports the classification of the Siphonales with the Chlorophyceae, the Heterosiphonales (Vaucheriaceae) and the Heterotrichales (Tribonemataceae) with the Xanthophyceae or Heterokontae.

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A MICROSPORIDIAN INFECTION OF THE DIGESTIVE TRACT OF THE WINTER FLOUNDER, PSEUDOPLEURONECTES AMERICANUS[1]

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Linton (1901) reported sporozoan infections in two small winter flounders, *Pseudopleuronectes americanus,* taken from Katama Bay, Martha's Vineyard, and examined at Woods Hole on August 28, 1900. His account, although brief, is adequate for recognition of the parasite and reads (p. 487), "The walls of the intestine of one throughout almost the entire length and of the other for a short distance were completely covered with sporocysts. The cysts were irregular where crowded together; where not crowded together, which was in but few places, they were elliptical or spherical, of various sizes, but comparatively few reaching 1 mm. in diameter and none much exceeding that. Spores oblong-ovate about 0.003 mm. in length and 0.0015 mm. in diameter. Intestine where affected was chalky-white in color." The accompanying figure shows a "Piece of intestine of *Pseudopleuronectes americanus,* serous coat covered with cysts due to sporosperms (*sic*)." There was no attempt at identification of the parasite, but Linton recognized that it was distinct from another, reported in the same publication (pp. 438 and 439), found in the muscles of the back and sides of the herring, *Clupea harengus,* and the alewife, *Pomolobus pseudoharengus.*

The latter species was identified as a myxosporidian and almost one-half of the young fishes were infected. Tyzzer (1900) reported the discovery and prevalence of this infection in young *P. pseudoharengus;* Auerbach (1910) assigned the species to the genus *Chloromyxum* Mingazzini, 1890; and Hahn (1917) proposed the specific name, *Chloromyxum clupeidae.* The allocation to *Chloromyxum* was based on the spore, which has a quadrilateral apical end and bears four polar capsules. Kudo (1920, p. 94) examined the slides prepared by Tyzzer, and others made from various species of fishes, and reported the infection in *Clupea harengus, Pomolobus pseudoharengus, P. aestivalis, P. mediocris, Brevoortia tyrannus, Stenotomus chrysops,* and *Tautogolabrus adspersus,* taken at Woods Hole. The parasites from the muscles of these fishes were regarded as specifically identical and referred to *Chloromyxum clupeidae* Hahn, 1917.

A third sporozoan was reported by Linton (1901; p. 455) from the liver of the butterfish, *Poronotus triacanthus* (syn. *Rhombus triacanthus*). The cyst was white and globular, about 1.5 mm. in diameter; when compressed it liberated immense numbers of spores, often aggregated in globular or oblong clusters, as large as 0.02 mm. in diameter. The spores were short and thick, with bluntly rounded ends, about 0.0025 mm. in length and a little less than that in breadth and thickness.

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The parasite is obviously a microsporidian and Woodcock (1904; p. 54) regarded it as a species of *Pleistophora*.

A fourth sporozoan was observed by Linton (1901; p. 433): an enormous number of small elliptical bodies, 14 by 6 microns, were found in the intestinal contents of a sting-ray, *Dasyatis centroura*. Since the spores were in the lumen of the gut, it is apparent that they were ingested in food and were parasitic in some animal other than the ray.

The parasites reported by Linton from the wall of the digestive tract of *P. americanus* are similar to and possibly identical with others reported about the same time from flat-fishes of Europe. Hagenmüller (1899) observed the infection in at least one-half ("18 fois sur 30") of the small fishes, *Flexus passer* Moreau (= *Pleuronectes passer*) from littoral pools in the area of Endoûme, Bouches-du-Rhône, France. The parasite was named *Nosema stephani* in honor of M. Pierre Stephan, who first found the cysts and called them to the attention of the author. He wrote (p. 837, "Cette Myxosporidie appartient au genre *Glugea* Thélohan, aujourd'hui *Nosema;* elle infeste, sous forme d'infiltration diffuse ou de kystes, les parois du tube digestif . . . L'infiltration diffuse représente plus particulièrement un mode de pullulation endogène, tandis que les kystes assurent la dissémination du parasite à l'extérieur. Kystes et amas d'infiltration s'observent depuis la partie supérieure de l'oesophage jusqu' à l'extrémeté du rectum, logés dans les tissus ou simplement recouverts par le péritoine. Il n'existe ni amas ni kystes dans le parenchyme d'aucun organe, rein, rate, foie, coeur, etc. Cependant, sous le péritoine à la surface du foie et dans les replis péritonéaux où cheminent des vaisseaux, les kystes sont assez nombreux; j'en ai trouvé jusque sur le conduit cholédoque près de son abouchement avec l'intestin. Dans la paroi intestinale, les kystes siègent dans les couches musculaires et surtout dans la couche conjonctive. J'en ai vu jusque dans la charpente conjonctive des replis de la muqueuse et des villosités, mais jamais, non plus que d'infiltration diffuse, dans la couche épithéliale de l'intestin.

"Ces kystes apparaissent à l'oeil nu comme de petits grains d'un blanc de lait, ovoides ou plus rarement sphériques, ne dépassant guère 1 mm. en diamètre, n'atteignant même pour la plupart que quelques dixièmes de millimètre, ou moins encore." Hagenmüller discussed the formation of the cyst and concluded that the membranous wall is produced by the host as a reaction to invasion by the parasite.

A similar and presumably identical species was reported by Johnstone (1901) from the plaice, *Pleuronectes platessa,* taken in the Irish Sea along the coast of Lancashire. The author recognized the parasite as a protozoan, probably a sporozoan, but further identification was not attempted. The infection was limited to the digestive tract and the intestine, from the pylorus to the anus, was thickened and superficially looked like a ripe ovary. The external surface was studded with small, round, white, opaque bodies; the internal surface was disposed in irregular, longitudinal folds, covered with projecting, round white bodies; the lumen was reduced and the mucosa often lost; and the wall measured 3 to 4 mm. in thickness. The cysts were about 0.60 mm. in diameter, with a capsule composed of an outer cuticular and an inner fibrous layer. The spores were oval with a maximum length of 0.005 mm. Figures portrayed the gross appearance of the intestine, the structure of the wall, and the form of the spores.

Woodcock (1904) described a second infection in the digestive tract of the plaice, *P. platessa,* taken near Plymouth, England, and discussed the Myxosporidia in flatfishes. For him, the Myxosporidia Bütschli, 1881 were "characterized (a) by the fact that reproduction by spores goes on throughout the growing or 'trophic' period, and (b) by the complicated process of spore-formation and the nature of the spores." The group was, thus, the equivalent of the Neosporidia Schaudinn, 1900 and the Cnidosporidia Doflein, 1901, and included the Microsporidia Balbiana, 1882. Woodcock reviewed the papers by Hagenmüller, Linton, and Johnstone; he noted that the infection reported by Johnstone was a "ripe, well-matured one," extensively distributed, whereas the infection he studied was only a slight and limited one, from a fish that superficially was quite healthy in appearance. In this specimen, the gut showed little oval patches, 1.00 mm. in diameter, usually projecting slightly on the outer, coelomic side together with other small pyriform appendages, 1.50 to 2.00 mm. in length, attached to the gut by the narrow end. These enlargements were all on the side of the gut to which the mesentery was attached, and in which the blood vessels ran. The functional activity of the intestine was not impaired; the mucosa was intact and normal in appearance. Woodcock compared sections made from the lightly infected intestine with others made from material sent by Dr. Johnstone. He discussed endogenous multiplication ("multiplicative reproduction" of Doflein) in young forms, the spread of the infection into neighboring tissue by diffuse infiltration and the formation of cysts and pseudocysts. He stated (p. 57), "Quite probably 'multiplicative reproduction' is, here, simply a separation of the pansporoblast rudiments, as daughter individuals. Indeed the whole nature of diffuse infiltration in *Glugea* seems to me to support this idea. There is no question of the individual parasites attaining size, still less of any continuity of a protoplasmic mass ramifying in and between host's tissue-cells. It is far rather a cell-infection, visible, when ripe, as separate clumps of spores, each formed from, and representing, one pansporoblast, and either still surrounded by a hypertrophied host-cell, or else free, but only owing to the latter's breakdown." The infections reported by Hagenmüller, Linton and Johnstone were referred to the same species, here designated as *Glugea stephani* (Hagenmüller, 1899) Woodcock, 1904.

Stempell (1904) studied the development of *Nosema anomala* Moniez, 1887, a species from the connective tissue in subcutaneous loci and in the gut-wall, liver, and gonads of the fresh-water stickleback, *Gasterosteus aculeatus.* This species had been transferred by Gurley (1893) to the genus, *Glugea* Thélohan, 1891. Stempell noted that recent investigations had disclosed a series of protozoan species in which the life-cycles consisted of a limited period of vegetative, asexual multiplication, after which different, *"geartete"* forms appear, whose further multiplication is conditional on the conjugation or copulation of two individuals. After citing essentials of these investigations he stated (p. 31), "Bedenkt mann dagegen, dass die allgemeinen Grundzüge der Entwicklung, soweit sie sich feststellen liessen, in allen Fällen dieselben sind, so darf man wohl mit Recht schliessen, dass sämtliche beschriebenen Parasitenformen der Spezies *Nosema anomalum* Monz. angehoren. In der Tat, ein treffender Name für eine so variable Spezies!" Accordingly, he returned the species, *anomala,* to the genus *Nosema.* In this species he reported growth of the protoplasmic masses with rapid, asexual multiplication of nuclei, followed by the differentiation of sporonts, the admitted progenitors of the sexual generation. His account reads (p. 33), "Wir sehen, wie in der enzystierten Parasitenmasse zunächst ein Wachstum des Protoplasmas und eine starke Vermehrung der Kerne auf rein ungeschlechtlichem Wege erfolgt, wie sich aber schon sehr bald aus dieser vegetativen Parasitenmasse die als Vorfahren der Geschlechtsgeneration aufzufassenden Sporonten differenzieren. Nur dadurch unterscheiden sich die vorliegenden Microsporidien und so viele phänozyste Myxosporidien von der Mehrzahl des anderen Sporozoen, dass diese Geschlechtgeneration durch endogene Knospung im Körper der vegetativen Individuen entsteht."

Weissenberg (1911) reported that about 2% of the smelt, *Osmerus eperlanus*, taken from sources near Berlin and from inlets of the Baltic Sea, were infected with a microsporidian parasite, similar to but distinct from *Glugea anomala*, which he described as a new species, *Glugea hertwigi*. He observed no difference in the infections of fishes from fresh and salt water. In a second paper, Weissenberg (1913) reviewed the work of Stempell (1904) and other authors on microsporidian species and reported on the life-cycles of *G. anomala* and *G. hertwigi*. Since the time of Pasteur it has been known that certain microsporidians invade the ovary and penetrate the ova, with hereditary transmission of infection. Stempell described such infected ova, but Weissenberg (1913) declared the evidence was not convincing. To test the matter, he raised sticklebacks, *Gasterosteus aculeatus*, from eggs. When the yolk-sacs were resorbed he fed small copepoda and daphnids but the fishes did not grow. Fine emulsions of spores were added to the water, but no infection resulted. With other fishes raised in aquaria but fed plankton with an abundance of plant and animal food, growth was good and two young sticklebacks daily were fed plankton mixed with an emulsion of spores. Three weeks after the beginning of the experiment, one of the fishes had a *Glugea*-cyst, 300 microns in diameter, on the wall of the throat. This result demonstrates that a fish raised in the laboratory became infected and provides information on the rate of cyst formation. Weissenberg concluded (p. 157), "Wenngleich die oben dargelegten Befunde bezüglich der Entwicklungsprozesse von *Glugea anomala* in zahlreichen Punkten von den Ergebnissen der Voruntersucher abweichen, so gelange ich doch zu der gleichen Gesamtauffassung wie die älteren Autoren, insbesonder Stempell. Auch nach meinen Befunden kommt *Glugea* ein grosser eigener Plasmakörper mit zahlreichen vegetativen Kernen zu. Die ganze Cyste gehört zum Protozoon. Wirtszellen oder hypertrophische Wirtskerne sind am Cystenaufbau nicht beteiligt." In a subsequent paper, Weissenberg (1921) discussed the problem whether or not the large Plasmakörper, with its large vesicular nuclei, is derived from host tissue or is of protozoan origin. After presenting new evidence he concluded (p. 420), "An der Wirtsgewebsableitung des Plasmakörpers und der bläschenförmigen Kerne der *Glugea*-Cysten kann nun nicht mehr gezweifelt werden. Aufgabe künftiger Forschung wird es sein, die Art der phagocytenartigen verstreut im Bindegewebe auftretenden Fischzellen, die somit den Mutterboden für die *Glugea*-Cysten abgeben, genauer zu eruieren."

Meanwhile, Mavor (1915) reported that about 50% of the *Pseudopleuronectes americanus* examined in the summer and autumn of 1910 at Woods Hole, Mass., were infected with *Glugea stephani*. He also found *Osmerus mordax* at Woods Hole frequently infected with a microsporidian, apparently *G. stephani*. These findings are in marked contrast to others made in the summer of 1912, when no infections were found on examination of 82 *P. americanus and* 22 *O. mordax* taken in the region of St. Andrews, New Brunswick. Kudo (1924) suspected that the parasite of *O. mordax* was *Glugea hertwigi.*

Schrader (1921) found 28% to 53% of the smelts, *O. mordax* from lakes in New Hampshire, and 1.5% to 16% of those from the coast of Maine were infected with a species which he identified as *O. hertwigi* Weissenberg, 1911. The intestine was the primary seat of infection although cysts were present in the liver and gonads. The cysts ranged in size from microscopic to 3 mm. in diameter, but were similar in size in each fish. The highest incidence of infection was in immature fishes, about 10 cm. long; adult fishes were rarely parasitized and Schrader predicated that the majority of infected fish die while immature. Unlike *G. anomala, G. hertwigi* was regarded as specific for smelts since other fishes in the same area were not infected. Furthermore, connective tissue and muscles were not infected, which apparently served to distinguish *G. hertwigi* from *G. anomala.*

Reichenow (1929) described *Glugea stephani* from infections of *Pleuronectes limanda* at Helgoland. He found white cysts, 0.5 mm. in diameter, in the submucosa of the intestine and reported (p. 1099), "Die Parasiten bilden zuerst Infektionsherde in der Darmwand, die von Hagenmuller und Woodcock als Zustand diffuser Infiltration (vgl. S. 1046) bezeichnet werden. Die Parasiten haben jedoch keinen interzellulären Sitz, vielmehr befallen sie im Laufe ihrer Vermehrung zahllose benachbarte Zellen (entweder Bindegewebszellen oder vielleicht Leukocyten, die sich an der Infektionsstelle ansammeln). Um den ganzen Herd herum bildet sich eine dicke Bindegewebskapsel, und so entstehen die Cysten, deren Inhalt also in diesem Falle nicht durch eine einzige Riesenzelle, sondern durch viele infizierte Zellen dargestellt wird. Die fertig ausgebildeten Cysten findet man hauptsächlich von ungeheuren Sporenmassen erfüllt, zwischen denen verstreut Zell- und Kernreste vorkommen. Eine paarige Anlage der Sporen, welche die Stellung dieser Art zu der Gattung *Glugea* begründen würde, ist von keinem der Untersucher beschrieben worden. Ich habe in dem von mir beobachteten Falle eher den Eindruck gewonnen, dass die Sporen einzeln entstehen, so dass die Art also zu *Nosema* zu rechnen wäre. Doch wird sich dies erst bei Beobachtung früherer Infektionsstadien, die übersichtlichere Bilder geben, entscheiden lassen. In meinem Falle, in dem die Cysten dicht gedränkt in der Darmwand sassen, war die Schleimhaut auf weite Strecken völlig abgestossen; es ist daher zu vermuten, dass die Fische an starken Infektionen zugrunde gehen."

Recent accounts have added little information on microsporidian infections of fishes. Bond (1938) identified cysts found in sections of the stomach of *Fundulus heteroclitus* taken in Chesapeake Bay as *G. hertwigi,* but the determination may not be correct. Fantham *et al.* (1941) described an infection in the hindgut of a specimen of *O. mordax* taken from Lake Edward, Quebec, and listed the parasite as *G. hertwigi* var. *canadensis.* Also, they reported *G. stephani* in the submucosa of the intestine of *P. americanus* and *Limanda ferruginea,* taken near Halifax, Nova Scotia; *L. ferruginea* was recognized as a new host of the parasite. Haley (1952) described a severe epidemic of microsporidiosis in *O. mordax* in Loon Pond, Gilmanton, New Hampshire, and 16 of 20 *O. mordax* from the Oyster River taken at

Durham, N. H., were infected by the same species, which he identified as *G. hertwigi.*

The Microsporidia are chiefly parasites of invertebrates, especially crustaceans and insects. The classification of the Microsporidia or Microspirida is based primarily upon the form and structure of the spores and to a lesser degree upon differences in the details of sporogenesis. The parasites of *P. americanus* belong to the family Nosematidae, characterized by small, oval or ovate spores, each with one polar filament. The genera are distinguished by the number of spores that are produced by each sporont. According to Poisson (1953), in *Nosema* each sporont develops into a sporoblast and produces a single spore; in other genera the numbers of spores produced are: *Glugea* Thélohan, 1891 and *Pérezia* Léger et Duboscq, 1909, two spores; in *Gurleya* Doflein, 1898 and *Pyrotheca* Hesse, 1935, four sporoblasts and four spores; but in *Stempellia* Léger et Hesse, 1910, the numbers of spores produced are: *Glugea* Thélohan, 1891 and *Pérezia* Léger et Duboscq, number varies from 8 to 32; and in *Plistophora* Gurley, 1893, each sporont (pansporoblast) produces more than 16 spores. It is generally believed that the microsporidia are narrowly host-specific. According to Poisson (1953) some 40 species in the genera *Plistophora, Glugea,* and *Nosema* occur in fishes and one species, *Glugea danilewsky,* occurs in the muscles and connective tissue of *Rana fusca, Emys orbicularis, Natrix natrix* and other hosts. If this determination is correct, the distribution of *G. danilewsky* belies the opinion that species of *Glugea* are host-specific.

The life-history of the Microsporidia, as conceived by Debaisieux (1928), comprises two distinct phases: a multiplicative stage, schizogony, beginning with the liberation of the uninucleate or binucleate sporoplasm or planont from the spore and its entry into a host-cell, and sporogony, a spore-forming stage, in which sporonts produce sporoblasts that give rise to resistant spores, the infective agents that serve for dispersal of the parasite and the infection of new hosts. According to Kudo (1924, p. 34), "No intermediate host animals have up to date been found for Microsporidia. The infection of a new host animal takes place when the latter ingests spores of a specific microsporidian capable of germinating in its gut." A similar statement was made by Dogiel, Petrushevski and Polyanski (1961) but no reference to experimental evidence was cited.

It is generally agreed that the life-cycle of the microsporidian involves sexual phenomena but there is wide disagreement concerning the location in the cycle where meiosis and syngamy occur. Meiotic phenomena have never been observed in the Microsporidia and syngamy has been reported by autogamy of nuclei in the sporoplasm before or after release from the spore, and also by nuclear fusion preceding sporont formation. Writing on sexual phenomena in Protozoa, Hall (1953, p. 80) stated, "A reduction of the chromosomes to the haploid number may occur in gametogenesis (*gametic meiosis*), in an early division of the zygote (*zygotic meiosis*), or in one of the pregamic divisions in conjugation (*conjugated meiosis*). The type of meiosis varies in different Protozoa. Available data indicate that the Heliozoida, Foraminifera, Cnidosporidia, and Ciliophora are diploid throughout most of the life-cycle." An opposite opinion was stated by Cheissin and Poljansky (1963, p. 343), "In the life-cycles of the Sporozoa the alternation of sexual process and sporogony or that of sexual process, sporogony and repeated asexual multiplication by means of schizogony occurs. All the developmental stages but zygote are haploid ones because the meiosis usually appears during the process of sporogony followed by formation of sporozoites." The statement by the Russian authors apparently is based on the situation in the malarial parasites, but the Microsporidia are distinct from the Haemosporidia and the life-cycles may be quite different. Indeed, Kudo (1944, p. 50) reporting on the life-cycle of *Nosema notabilis* Kudo, 1939, stated, "Schizogony is by binary fission. No sexual process has been observed in the development of *Nosema notabilis*."

The small size of the amoeboid stages and of the spores, usually less than 4 microns in length, together with the inability to obtain early stages by controlled experimental infections of fishes, has made it impossible to describe the developmental cycle of these microsporidian species with assurance. The time and place of chromosome-reduction in meiosis and of syngamy are controversial. Cells with two nuclei may represent a stage before fusion of gametes or the first division of a zygote. Specific distinctions are often precarious and even generic diagnoses are unsatisfactory. In his monographic treatise, Poisson (1953) stated (p. 1043), "Mais bien des espèces de Microsporidies sont insuffisamment étudiées; trop d'espèces ont été décrites comme nouvelles parce qu'elles étaient trouvées dans des hôtes nouveaux. D'après Steinhaus et Hughes par example, *Nosema destructor* S. et H, a été observée chez au moins 10 espèces d'Insectes apparentant à trois groupes différents: la chenille de *Gnorimoschema operculella* (Zeller) (Lépidoptère), des Hyménoptères, des Névroptéroides. Il est donc des Microsporidies qui ne manifestent qu'une spécificité toute relative. D'autre part, les caractères distinctifs utilisés pour séparer les espèces, et même les genres, n'offrent peut-être toujours la précision désirable et certains genres, tels les genres *Nosema*, *Plistophora*, *Glugea*, *Perezia*, devront être revisés."

Microsporidian infections of *Pseudopleuronectes americanus* have long been known by members of the staffs of the New York Aquarium and the New York State Conservation Department, but precise and detailed records of incidence and intensity are not available. Dr. Ross F. Nigrelli, at the Aquarium of the New York Zoological Society, has observed the frequent occurrence of the parasite in fishes of the New York area and Mr. John C. Poole of the Conservation Department reports that the infection has a "spotty" distribution, *e.g.*, in one year over 25% of the young of that year taken in Shinnecock Bay were infected and no infection was found in the same location the following year.

Materials and Methods

The present investigation was begun in the summer of 1961 and has been conducted more or less continuously since that time. Over 1000 fishes, *P. americanus*, taken from different locations in New England, have been inspected for microsporidian infection. Data have been compiled (Tables I–VI) on the number of fishes examined, the time of year and area where they were caught, their size, sex, and the incidence and intensity of infection. Fishes taken on Georges Bank, off Yarmouth, Nova Scotia, were caught on the August, 1963, cruise and those from Nantucket shoals on the April, 1964, cruise of the Albatross IV. Records denote the organs involved and the extent of infection. Winter flounder are present from April to November in Woods Hole harbor. The stomachs and intestines of 751

TABLE I

Incidence of microsporidian infection in winter flounder from Woods Hole Harbor in 1962

Month	Number examined	Number infected	Per cent infection
April	80	3	3.4
May	67	2	2.3
June	86	1	1.2
July	102	7	6.9
August	156	6	3.8
September	86	0	0.0
October	125	3	2.4
November	49	4	8.2
	—	—	—
Total	751	26	3.5

fishes were removed and preserved for food analysis, and patent sporozoan infections were noted. Very light infections may have been missed, so the recorded intensity is minimal. Analysis of the stomach-contents was made to determine the kinds and amounts of food ingested. In November, 1964, about 300 young fishes that measured from 40 to 110 mm. in length were taken in Lake Tashmoo, Martha's Vineyard, where the infection-rate was known to be high. Eighty-five of these fishes, which died at the time of collecting or a few hours later, were examined and the results are given in Table VI. Heavy infections included those where the infil-

TABLE II

Length-distribution of microsporidian-infected winter flounder compared with that of all winter flounder examined, in Woods Hole Harbor during 1962

Length (cm.)	Number of fish		Length (cm.)	Number of fish	
	Total	Infected		Total	Infected
12	1		28	64	6
13	1		29	70	
14	1		30	66	2
15	6		31	70	1
16	3		32	66	1
17	1		33	48	2
18	7	1	34	45	1
19	11	1	35	33	2
20	11		36	20	
21	7		37	16	
22	15		38	6	1
23	19	2	39	6	
24	18		40	1	
25	33	2	41	2	
26	48	2	42	2	
27	51	2			
				—	—
				751	26

TABLE III

Mean weights of infected and non-infected winter flounder from Woods Hole Harbor in 1962

Mean length cm.	Infected		Non-infected	
	Number	Mean weight	Number	Mean weight
July–August				
males				
23	2	193	5	211
30	2	366	9	351
33	1	443	7	439
34	1	402	4	513
females				
18	1	76	—	—
23	1	150	3	165
27	1	232	6	272
28	4	278	10	293
October–November				
males				
19	1	70	1	64
26	2	194	6	223
females				
28	1	280	9	270
31	1	404	9	368
35	1	541	8	551
38	1	652	3	774

tration was massive and the gut was partly or largely destroyed; light infections included those with from one to 20 cysts in the wall of the intestine. By the time that cysts are formed the infection is already well established.

In Table VI, the winter flounders less than 100 mm. in total length were of the 1964 year class, *i.e.,* less than a year old. Those of 100 mm. or more in length probably were of the 1963 year class, but final age-determination was not made.

Since the microsporidiosis is located primarily in the intestine, and the infective agent was presumably taken in with food, the stomach-contents of 751 fishes from

TABLE IV

Amount of food in stomachs of infected and non-infected winter flounder from Woods Hole Harbor in July–August, 1962

Month	Infected			Non-infected		
	Number	Mean length cm.	Food grams	Number	Mean length cm.	Food grams
July	7	28	1.25	102	30	2.19
August	6	26	1.02	156	27	0.82

TABLE V

Incidence of microsporidian infection in winter flounder from different New England fishing grounds

Location	Georges Bank		Off Yarmouth, Nova Scotia		Nantucket Shoals		Off Plymouth, Mass.	
Length cm.	Total number	Number infected	Total number	Number infected	Total number	Number infected	Total number	Number infected
11–15	1	0	0	0	0	0	0	0
16–20	1	0	1	1	0	0	0	0
21–25	0	0	0	0	16	1	1	0
26–30	1	0	0	0	25	1	0	0
31–35	6	0	2	0	53	11	12	1
36–40	6	0	3	0	23	7	5	2
41–45	16	0	2	0	8	1	1	0
46–50	2	0	0	0	1	0	0	0
51–55	3	0	0	0	0	0	0	0
56–60	1	0	0	0	0	0	0	0
61–65	1	0	0	0	0	0	0	0
Total	38	0	8	1	126	21	19	3
Per cent infected	0		1.2		16.7		15.8	

Woods Hole harbor have been examined in an attempt to discern the source or sources of the infective agent or agents. Also, since Microsporidia are presumed to be one-host parasites, microsporidian cysts from winter flounder gut-wall embedded

TABLE VI

Incidence of microsporidian infection in small winter flounder from Lake Tashmoo, Martha's Vineyard

Length (mm.)	Degree of infection		
	Heavy	Light	None
41– 45	2	0	0
46– 50	4	0	1
51– 55	6	1	1
56– 60	6	3	3
61– 65	2	3	4
66– 70	0	2	7
71– 75	1	6	8
76– 80	1	2	4
81– 85	0	2	4
86– 90	1	4	1
91– 95	0	0	2
96–100	0	0	0
101–105	0	0	2
106–110	0	0	2
Total	23	23	39

Per cent infected, 54.1.

in pieces of clam, *Mercenaria mercenaria,* have been fed to other winter flounder kept in aquaria in attempts to induce experimental infections. Cysts were fed to two fish in the summer of 1962. They were examined six weeks later and there was no evidence of infection. Five fish were fed cysts in November, 1964. Three were examined in April, 1965, and the other two were autopsied in June, 1965. No infection resulted from these experiments. It appears that direct infection does not occur, that either the sporoplasms do not emerge from the spores or they fail to invade the intestinal epithelium.

Tissues from natural infections were fixed in different fluids, cut in serial sections at 5 and 10 microns in thickness, and stained for particular effects. Haematoxylin and erythrosin were used for general purposes and routine pathological staining. Heidenhain's iron technique was employed on thin sections for cytological details and azan trichrome for special histology.

Results

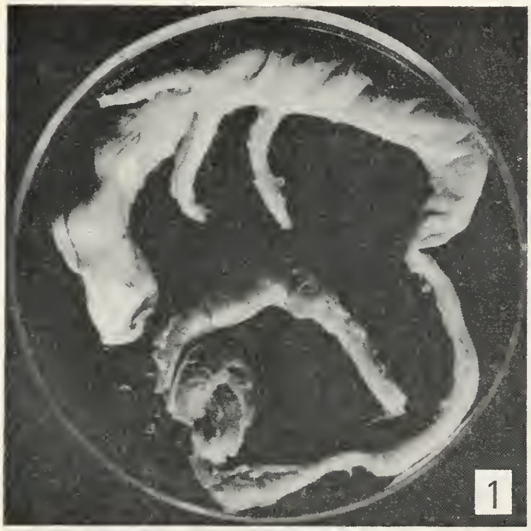
The incidence of infection in 751 fishes taken in the Woods Hole harbor and examined each month, April through November, 1962, and data on the size and sex of the fishes are presented in Tables I–III. There was no apparent effect of seasonal or sexual differences. Table IV records the amount of food in the stomachs of infected and non-infected fishes of comparable sizes taken from Woods Hole harbor in July and August, 1962. There was no obvious relationship between infection and amount of food in the stomach. Stomach-contents of 386 fishes collected in weekly samples in September, October, and November, 1961, consisted by weight of algae, 42%; mollusks, 25%; polychaetes, 24%; crustaceans, 5%; and other (mostly unidentified), 4%. The results of analyses made in 1962 are similar, with less algae eaten in the spring and summer. No fish were found in any of the flounders examined, thus confirming the statements by Bean (1903), Breder (1929) and Bigelow and Schroeder (1953) that young flounders feed exclusively on algae and invertebrates, chiefly crustaceans and polychaete annelids. The account of Bigelow and Schroeder is very complete and includes the findings of Breder and Linton as well as their own observations. It is generally agreed that the small mouths of these flounders preclude the ingestion of fishes as food, and it appears certain that fish are of no significance in the diet of *P. americanus* in the Woods Hole area.

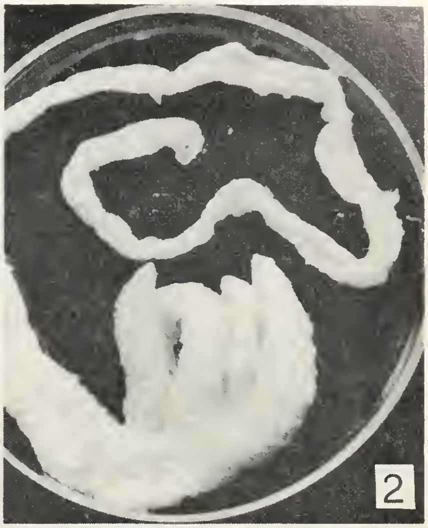
The incidence of infection in winter flounder of different sizes taken from different coastal areas and from Georges Bank, which is offshore, is presented in Table V. Although the number of fishes from Georges Bank is small, the absence of infection there may be significant. There is evidence that Georges Bank winter flounder are geographically isolated from those on inshore grounds and that they have no contact with the shore at any time during their lives. Results from the release on inshore grounds of over 10,000 tagged winter flounder indicate that only one was re-caught on Georges Bank (Perlmutter, 1947; Bigelow and Schroeder, 1953). Perlmutter also reported that winter flounder from Georges Bank have more fin rays than those from inshore grounds north and south of Cape Cod. Winter flounder from inshore subpopulations, on the other hand, are closely associated with the shore, spending their first year in estuaries and bays where much of the spawning occurs, and where infection may take place.

The data from the small fishes taken in November, 1964, at Martha's Vineyard (Table VI) are particularly interesting. In addition to the information presented in the table, 49 individuals in the same size-range died in the period November 20 to December 7, 1964. Nineteen of these fishes, mostly 65 to 85 mm. in length, were infected, with an incidence of 38.8%. Seven of the infections were heavy; 12 of them were light. Fifteen of the remaining fishes were killed March 12, 1965, and 8 of them, *i.e.*, 53.3%, were infected. Three of these infections were heavy; five were light. Inspection of the data from the 149 fishes examined shows that infection was greatest in small fishes. Almost all of the heavily infected ones were less than 80 mm. in length and some of them were less than 50 mm. in length. Since development of such massive infections must take some time, it is apparent that infection occurred very early in life, when the food consisted of small invertebrates. Comparison of the findings recorded in Tables II and VI, indicates strongly that fishes heavily infected during the first year of life do not survive into their second year.

The site of infection is primarily the wall of the intestine and pyloric ceca, but in moderate and heavy infections, other structures adjacent to or in contact with the gut may be involved. These include the bile-duct, liver, mesenterial lymph-nodes and the ovary. The infections observed were already well advanced and were manifest by cysts (Figs. 4, 5) embedded in the connective tissue of the organs affected. In larger fishes, most of the infections were light and apparently did not seriously affect the hosts. In light infections, the cysts were usually on the external wall of the intestine, but in heavy ones, the gut wall was largely supplanted by layers of cysts. In such instances the intestine had a chalk-white, pebbled appearance and the wall was rigid, thickened and hard. Photographs of intact normal and parasitized digestive tracts and of sections of the intestine and cecum of infected fishes portray the effects of massive infection. Figure 1 is of a normal digestive tract. In the specimen shown in Figure 2, the anterior end of the digestive tract is the principal site of infection, whereas in Figure 3, it is the rectal end of the specimen that is most heavily parasitized. Figure 4 is a photograph of a cross-section of the intestine shown in Figure 2, and Figure 5 is a photograph of a cross-section of one of the pyloric ceca taken from the specimen shown in Figure 2. The epithelium of the intestine in Figure 4 is denuded and the lumen of the cecum (Fig. 5) is almost occluded.

The cysts are spherical to oval unless deformed by pressure. They measure 0.6 to 1.0 mm. in diameter and the wall (Figs. 6, 7) is composed of laminated layers that have the structural appearance and staining reactions of the connective tissue of the host. In addition to those in the cysts, there are masses or strands of spores scattered about in the tissue of the gut wall, often associated with or paralleling blood vessels. The material at present available for study consists of relatively mature infections and the multiplicative phases have largely been completed. Rarely, near the wall of a cyst or in the intercystal areas there is a cell, which may be a pansporoblast, which contains a large number of bodies that color deeply with nuclear stains. Whether or not these structures are the nuclei of sporoblasts could not be determined. Below the connective capsule of the cyst there is often a narrow layer of stainable material, termed endoplasm by Woodcock, which contains large oval, apparently pycnotic, nuclei with fragmented chromatin and distinct nucleoli. Their presence suggests that the cyst is formed around a number of host-cells, whose cytoplasm has been consumed and whose nuclei persist below the wall of the cyst. The spores are oval to ovate, and when fixed and stained measure about 4 by 2.5 microns. Precise and accurate measurements of such minute and refractive bodies are difficult. The basal, wider end of the spore contains a vesicle





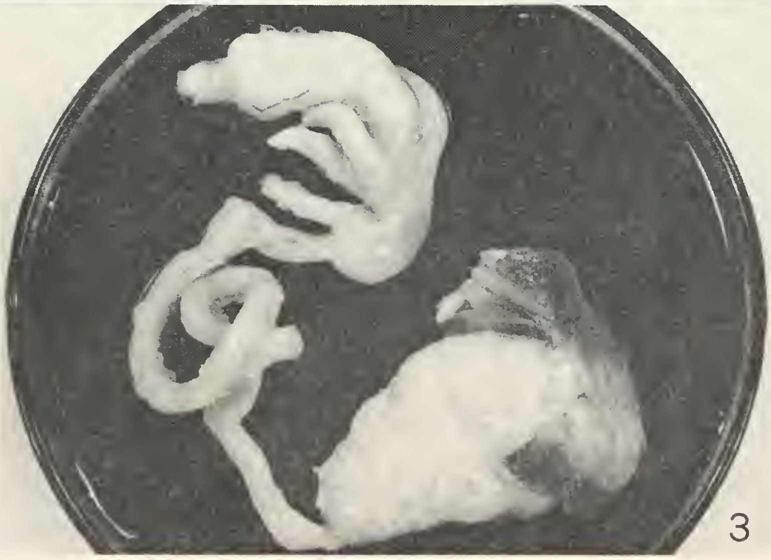
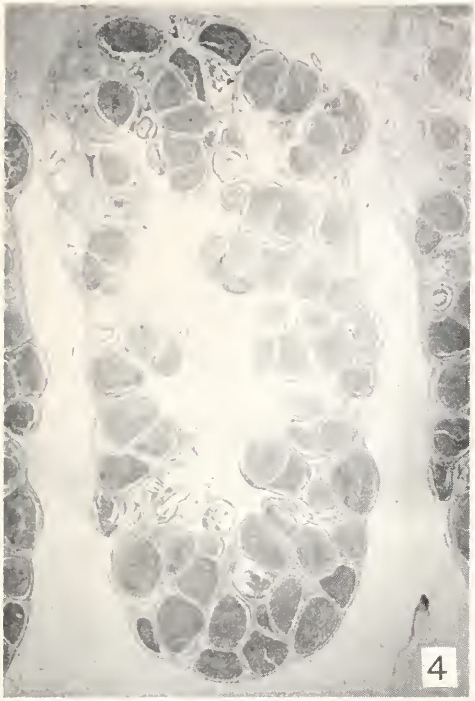


PLATE I

FIGURE 1. Digestive tract of *P. americanus,* normal condition, in a Petri-dish, 9.5 cm. outside diameter.

FIGURE 2. Pyloric ceca and intestine of infected fish, same magnification as Figure 1.

FIGURE 3. Digestive tract of infected fish, same magnification as Figure 1.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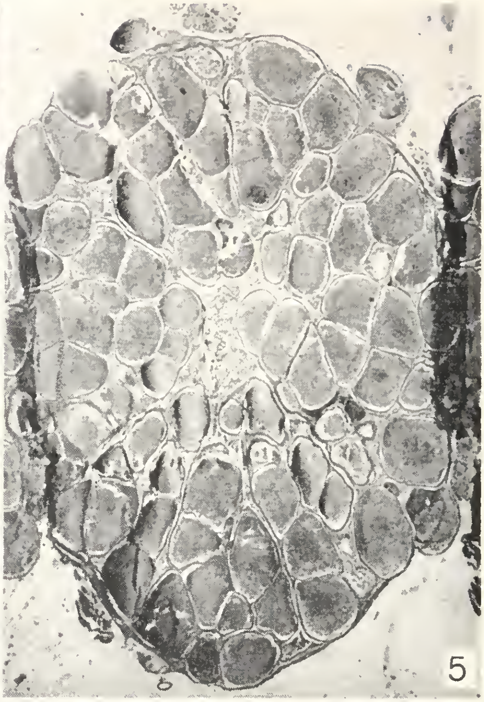
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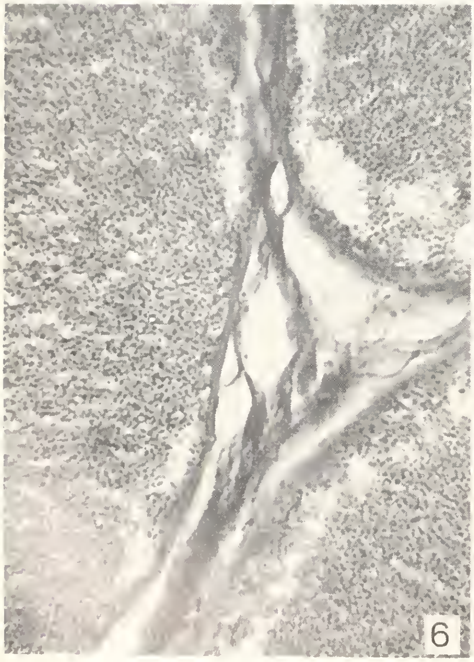
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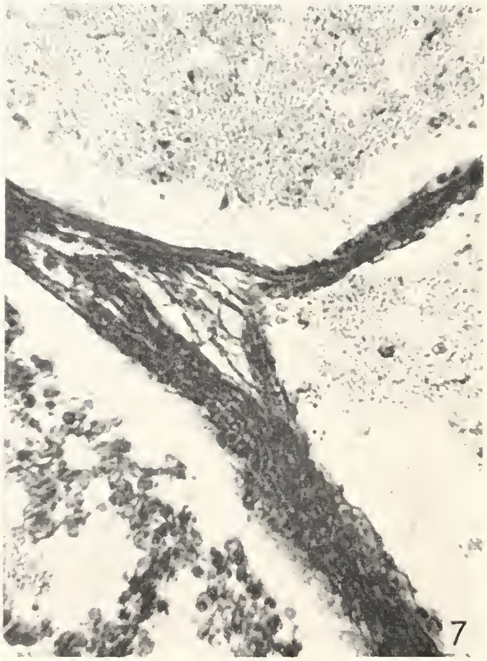
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PLATE II

that may occupy almost one-half the length of the spore. The apical end also contains a smaller vesicle, while the central portion contains a band of chromatic material, often in the form of strings of particles or granules, and a single strand extends to the apical end of the spore.

DISCUSSION

The most comprehensive account of the Microsporidia is the monograph by Kudo (1924). He gave a review of morphology and life-cycles, with a description and taxonomic survey of all previously described species. In a later study, Kudo (1944) stated (p. 38), "The early phases of the development of Microsporidia have not been seen in many species. In a few instances of experimental infection, certain portions of the development have been seen, but in no case has observation in life been carried through." It is generally agreed, however, that the life-cycle of a microsporidian species consists of two distinct phases or stages: a multiplicative stage, schizogony, and a spore-forming stage, sporogony. In the multiplicative phase, cell division is rapid and according to certain investigators it may result from binary fission following nuclear division or multiple fission if nuclear division is rapid and cytoplasmic division is delayed. It has been suggested (Kudo, 1924) that in certain species the schizonts (meronts of Stempell, 1902) are not motile and as a consequence that the progeny of a sporoplasm remains in the host-cell and that all the spores formed in that cell are derived from the initial parasite. But usually the infection is invasive, with diffuse infiltration of tissue, and such a condition could result from either the penetration and dispersal of enormous numbers of planonts or by the liberation of schizonts and their ingestion by leukocytes or macrophages which accumulate at sites of inflammation and which could transport the schizonts to other areas and extend the infection. At the end of the schizogonic phase, sporonts are formed but the factors involved and details of the phenomena which result in the formation of sporonts are equivocal. Supporting the observations of Weissenberg (1914), Debaisieux (1920) and Guyénot and Naville (1922), Kudo (1946, p. 162) stated, "In the Microsporidia, autogamy appears to initiate the spore-formation at the end of schizogonic activity."

In the present study, the inability to obtain experimental infection of fishes has precluded observations on the multiplicative phases of the life-cycle. But this inability has raised important and perplexing problems. Since fishes become infected when only 50 mm. in length and when the food consists of small invertebrates, it seems probable that a second or intermediate host may be required in the life-cycle of the parasite. Such an invertebrate may be merely a paratenic or transport host, which ingests spores from a dead fish and is then eaten by a small flounder, or it may be essential in the completion of the life-cycle of the parasite. Since

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FIGURE 4. Photomicrograph of cross-section of the intestine shown in Figure 2. Note lack of digestive epithelium and disintegration of the gut wall.

FIGURE 5. Photomicrograph of cross-section of one of the pyloric ceca shown in Figure 2. The infection is more intense in this area than in the intestine.

FIGURE 6. Photomicrograph of section of pyloric cecum, greater magnification, to show connective tissue capsular wall of the cyst and number of spores.

FIGURE 7. Photomicrograph of section of pyloric cecum, showing walls of cysts and adjacent nuclei and cells.

small crustaceans are carnivorous and constitute a considerable part of the food of small fishes, they become suspect. According to the account of Frederick E. Smith, (The Benthos of Block Island Sound: Ph.D. thesis, Yale University, 1950, 213 pp. and appendices), 75% of the food of the winter flounder consisted of amphipods and 43% of the amphipods were *Leptocheirus pinguis.*

Other questions also arise: why are certain infections mild while others become massive? Do older fishes develop resistance to infection and restrict the invasive activity of the parasite? Why do the cysts manifest such uniformity in size? Finally, in view of the statements of Reichenow (1929) and Poisson (1953) that generic concepts are tenuous, what is the status of *Glugea* and does the species, *stephani,* belong in that genus? The answers to these and other questions await further investigation on the life-cycle of the species.

SUMMARY

A microsporidian infection of the blackback or winter flounder, *Pseudopleuronectes americanus,* has been investigated. It was first noted at Woods Hole, Massachusetts by Linton (1901) and may be identical with similar infections of European flounders reported by Hagenmüller (1899), who described the parasite as *Nosema stephani.* Woodcock (1904) transferred the species to *Glugea,* a genus erected by Thélohan (1891) to contain a parasite of the striated muscle in *Cottus scorpio* and *Callionymus lyra,* which he described as a new species, *Glugea microspora.* Gurley (1893) predicated that *G. microspora* is identical with *Nosema anomala* (Moniez, 1877), although he recognized *Glugea* as a valid genus, distinct from *Nosema.* In New England the infection is common in *P. americanus.* The incidence and intensity of infection in fishes of different sizes and from different geographical regions are reported, together with an account of the resultant pathology. Attempts to obtain experimental infection of fishes have not been successful and the life-cycle of the parasite remains unknown.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

1965

ABSTRACTS OF SEMINAR PAPERS

July 13, 1965

Suntanning. George Szabó, Madhukkar Pathak and Walter C. Quevedo, Jr.

The human skin contains three pigmentary substances, the carotenes, the hemoglobins and melanin. The amount of melanin varies from race to race, yet the population density of melanocytes (the pigment-forming dendritic cells of the epidermis) is the same in all human races. It follows from this that an increase in pigmentation of the same individual following exposure to ultraviolet radiation (sun or sun lamp) may not be due to an increase in the density of melanocyte population as claimed by earlier investigators.

We approached this problem by using two different experimental designs: (1) (Szabó and Pathak). We irradiated the forearm of human patients or ears of colored guinea pigs with a single dose of ultraviolet light, using a high energy source monochromator, delivering a minimal erythema dose equal to 35.6×10^6 ergs per 0.5 cm^2. The number of dopa-positive melanocytes was estimated by counting these cells in pure epidermal preparations, treated with DOPA reagent. No significant increase in the density of melanocyte population was observed, although the amount of pigmentation increased in the epidermis. Histochemical and biochemical tests revealed an increased tryosinase activity after 72 hours. (2) (Szabó and Quevedo) Non-exposed parts of human volunteers were irradiated repeatedly by commercial sun lamps. Biopsy specimens were also taken from one individual after several exposures to natural sun light. In both instances the density of dopa-positive melanocytes increased as the amount of melanin in the epidermis also increased.

These experiments show that the mammalian epidermis is able to increase its melanin content without recourse to an increase in the density of dopa-positive melanocytes. In natural conditions, however (exposure to sun), the pigmentary process may be combined with a mitotic activity of melanocytes.

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Cyclical synthesis of ribosomes in synchronous cell cultures. W. S. Vincent.

The advent of methods which bring about synchronously dividing cell populations has allowed biochemical studies of various metabolic events during the cell cycle. Studies of the times of enzymic synthesis and rate increases have led to the hypothesis that the control of the temporal sequence of biochemical events during the cell cycle may reside in the sequential translation of the linear sequence of genes on the chromosome. Evidence has also emerged that the ribosomal RNA cistrons are localized in a discrete area of a single chromosome, namely the nucleolus-associated chromatin. On the basis of the above conclusions, one might expect that analysis of RNA production during the cell cycle would demonstrate a localized burst of ribosomal RNA synthesis and ribosome assembly at some portion of the cell cycle.

This possibility was examined in synchronous cultures of the fission yeast, *Schizosaccharomyces pombe*, by P^{32}-labeling and sucrose gradient analysis of RNA isolated from purified ribosome-polysome preparations. Rates of RNA synthesis were measured by P^{32}, C^{14}-uracil and C^{14}-adenine.

The rates of RNA synthesis throughout the cycle are clearly cyclical, reaching a maximum at about 0.5–0.7 generation time (GT) and a minimum at about 0.3 GT. Ribosomal RNA synthesis and ribosome assembly are found to occur throughout the entire cycle at a constant rate, except for the period of 0.5–0.75 GT where the rate increases by a factor of 4 to 5. Within the limits of the precision of the experiments, it appears that one-half to two-thirds of the total new ribosome assembly occurs during this period, while the rest is evenly spread throughout the cell cycle.

This work was supported in part by grants from the National Science Foundation and the United States Public Health Service.

Action of bile salts on ATP-ase activity of mucosal homogenates from rat jejunum and ileum. Robert G. Faust and Shih-min Liu Wu.

Mucosal homogenates of rat jejunum and ileum (0.4 to 1 mg. dry wgt. of tissue) were incubated in a medium containing various concentrations of sodium taurocholate, sodium glycocholate, Na^+ and K^+, 50 m*M* Tris-HCl (pH 7.45), 0.4 m*M* $MgCl_2$ and 0.4 m*M* ATP at 37° C. for three minutes. The $Na^+ + K^+$ stimulated ATP-ase activity of both the jejunal and ileal homogenates was inhibited by 1 m*M* ouabain. Mucosal ATP-ase activity of jejunum was approximately three times greater than that observed with ileum. Both conjugated bile salts at 1 and 2 m*M* increased the ATP-ase activity of jejunal homogenates in the presence of $Na^+ + K^+$ and in the presence of low Na^+ concentrations and no added K^+. Although 2 m*M* sodium taurocholate increased the ATP-ase activity of mucosal homogenates from ileum in the presence of small quantities of Na^+ and no K^+ added to the reaction medium, sodium glycocholate, at 1 and 2 m*M*, inhibited ATP-ase activity under these conditions. On the other hand, the $Na^+ + K^+$ stimulated ATP-ase of mucosal homogenates from ileum was increased by 1 m*M* sodium glycocholate and 2 m*M* sodium taurocholate. However, the ATP-ase activity of ileal homogenates, under these conditions, was not increased when the sodium glycocholate concentration was raised to 2 m*M*. These bile salts at low concentrations have been previously shown by Faust and Wu to uncouple oxidative phosphorylation and to reduce ATP levels in rat jejunum and ileum.

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July 20, 1965

Neuroendocrine control of the crustacean hepatopancreas. Milton Fingerman and Yoshihiro Yamamoto.

The crustacean hepatopancreas performs secretory, absorptive, and storage functions. Removal of both eyestalks from the crayfish, *Procambarus clarki,* results in degeneration of this organ as seen histologically. The present investigation was undertaken to determine some of the physiological changes that accompany the morphological deterioration. A decrease in the ribonucleic acid (RNA) content of the hepatopancreas was found in eyestalkless specimens. Eyestalk extract slowed this loss and caused the reappearance of RNA after it had no longer been detectable. The total nitrogen per gram of tissue remained quite constant in intact unfed specimens but after the eyestalks were removed a progessive decrease occurred during the 13 days of observation. The pH of the gastric juice in intact individuals was 5.05 after one day of starvation and 5.30 after 10 days. The corresponding values for eyestalkless individuals were 5.30 and 5.88. The amylase content of the hepatopancreas in intact starved crayfish markedly increased over a 10-day period but in eyestalkless individuals only a slight rise was detected. In the gastric juice, however, the level of amylase in eyestalkless crayfish showed a drop after 10 days but a rise occurred in the juice of intact crayfish. Presumably, the amylase of the intact individuals spilled over from the hepatopancreas into the juice, although the crayfish were not fed, because of the large quantity that had accumulated; but in the eyestalkless crayfish not enough amylase could be produced to saturate the hepatopancreas. It is concluded from these experiments that a neuroendocrine product from the eyestalk is required for RNA synthesis by the hepatopancreas, and the RNA in turn is necessary for amylase synthesis.

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Intracellular aggregates and granules of Limulus gill cartilage. DELBERT E. PHILPOTT AND PHILIP PERSON.

Gill cartilage of *Limulus polyphemus* contains large amounts of lipid and glycogen when studied in the electron and light microscopes. Both perichondrium and adjacent cells, for several layers in depth, appear to be laden with the above substances in the form of lipid droplets, glycogen granules and admixtures of both (and other substances as well). Lipid droplets and glycogen particles are seen intra- and extracellularly. The osmiophilia of the lipid granules in electron micrographs varies from light grey to intense black. This variability is seen not only in different droplets, but within a single one. At times it is possible to discern a unit membrane surrounding the lipid droplets. Within many of the lipid droplets, pools or areas of glycogen are readily identified. In some aggregates, in addition to glycogen, rough-surface endoplasmic reticulum can be seen embedded in the lipid, and ribosomes are identifiable. Light microscope study of fresh-frozen sections of the tissue shows a variable metachromatic staining of the above lipid, etc. aggregates, the largest of which are readily identifiable in the light microscope. Both toluidine blue and methylene blue have been used for demonstration of metachromasia. In addition, glutaraldehyde-osmium-fixed tissue, embedded in Araldite-502, has been sectioned at 2 μ and stained with the above reagents. Some aggregates stain intensely metachromatic, some are faintly so, and some are either orthochromatic or do not take the stain at all, both in fresh-frozen and the plastic-embedded sections mentioned above. It is suggested that this tissue, in addition to serving as a valuable material for study of cartilage, may also provide a useful tool for studying interrelations between, and integrations of, lipid, carbohydrate and protein metabolism at the level of the electron microscope.

JULY 27, 1965

A biological rhythm in Euglena. JOHN D. PALMER.

During daytime low tides in the River Avon, in England, the exposed river banks take on a dappled green color. This color is due to the presence of enormous numbers of specimens of *Euglena obtusa* which emerge out of the black mud. Cell densities on the surface surpass 10^5 cells/cm.^{2}. Before the tide returns to cover the area, the cells re-burrow back into the mud and remain there during high tides and at night.

The cells in mud samples maintained in the laboratory in constant light and temperature—and away from the influence of the tide—continue to undergo these rhythmic vertical migrations for nearly one month; under these conditions the rhythm is *diurnal,* rather than quasi-tidal as it is in nature.

The form and amplitude (but not the period) of the daily rhythm in the laboratory are a function of the intensity of the constant illumination: the greater the light intensity, the longer the cells remain on the surface. Artificial darkening of the cells causes them to re-burrow at any point during the surfaced phase of their rhythm. Because of the various combined effects of light and darkness on the rhythm it is postulated that the vertical-migration rhythm is a secondary and unavoidable consequence of a more fundamental rhythm in photo-response.

The period of the rhythm in cells maintained in temperatures between 5 and 15° C. is virtually unaltered: it remains approximately 24 hours ($Q_{10} = \sim 1.0$). At 18.5° C. (the highest temperature available) the period is *lengthened* to 24.5 hours ($Q_{10} = \sim 0.94$). The existence of a temperature-compensating system associated with the rhythm is indicated by the latter temperature-coefficient, which suggests that *over*compensation has taken place.

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Negative slope Na-conductance in the surface structure of frog skin epithelium. BERND LINDEMANN.

The potential of 1 cm.^{2} frog skin was controlled electronically (clamping speed 1 msec.). The steady-state current voltage curve was found to be N-shaped for inward current, if Na was present in the outer bathing solution (1/10 Ringer). The coordinates of the peak in this curve are I_s (rheobase) and V_s (potential threshold). Vasopressin caused a large rise in I_s (increase of Na-conductance of skin surface) and only a small increase in V_s (Kutschera and Lindemann, 1965). When the Ca-content of the outer bathing solution was increased to 10 m*M*, I_s decreased, mainly by a 100-mV drop in V_s. The (V_s, I_s)-function is $V_s = V^* + I_sR_n$, where $V^* = I_sR^*$ is constant for any given $[Ca]_o$, and R^* is the Na- and vasopressin-sensitive surface resistance. For the series resistance R_n (inner barriers of epithelium) a constant value of 0.2–0.6 KOHmcm.^{2} (skin area) was computed. Apparently, the Na permeability of the outer skin surface decreases with increasing membrane potential when V^* is exceeded. This causes the fast potential rise (Finkelstein, 1964) under current clamp conditions. The positive exponential time course of this potential rise was found to be mostly determined by the steady-state negative slope Na-conductance divided by the membrane capacitance, rather than determined by the (faster) development of the negative slope conductance in time. In contrast, the potential time course of the falling phase (current clamp) is determined by the development in time of a non-specific conductance increase, which is initiated by and overlasts high membrane potentials (compare "anodal breakdown" in nerve, Hodgkin, 1947). It causes a distortion of the current voltage function of R^*, but the N-shape can to some degree be retained (notch in time course of potential drop). The distortion can partly be prevented by increasing $[Ca]_o$.

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Chemotaxis (?) of coelenterate sperm. RICHARD L. MILLER.

Previous work has shown that the sperm of the hydroids *Campanularia flexuosa* and *C. calceolifera* are attracted to the aperture of the homologous female gonangium. An active extract of *C. calceolifera* female gonangia can be obtained by treatment of the gonangia with polar solvents. This extract attracts the homologous sperm only.

Sixteen mm. motion pictures taken of the approach of the sperm of both species to the homologous female gonangium and, in the case of *C. calceolifera,* to a pipette injecting the isolated chemotactant, have been analyzed frame-by-frame. Where possible, every sperm in the field of view was followed and its speed determined. These analyses yielded the following results:

1. Sperm may or may not speed up when approaching the gonangium or pipette but slowing down is rarely seen and is usually associated with a turning movement. Increases in speed of as much as 400% have been measured.

2. Forty per cent to 60% of the turns made in the vicinity of a female gonangium (*C. flexuosa*) are decidedly non-random in nature and lead to entry. Ninety-five per cent to 98% of *all* trails within the range of effectiveness of the chemotactant show the sperm either entering the gonangium or striking the perisarc near the aperture.

3. If the isolated chemotactant (*C. calceolifera*) is injected into a sperm suspension, the sperm in the area activate and aggregate about the tip of the pipette. Free-swimming as well as thigmotactic sperm are affected in this way. Furthermore, thigmotactic sperm, the future paths of which can be predicted, deviate by turning toward the pipette when *entering* the area of aggregation.

These results indicate that the behavior of the sperm, if compared with the types classified by Fraenkel and Gunn (1940), cannot be an orthokinesis or a pure klinokinesis. Since randomness of turning is not a feature of the sperm behavior during attraction, the behavior is most likely a taxis, and may be considered a klino-chemotaxis.

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Effects of direct current passing through a model epithelial cell. Bernd Lindemann.

Consider direct current flow through well stirred ionic solutions 1, 2 and 3, which are in series and separated by membranes (o) and (i). Volume of solution 2 is constant and much smaller than of solutions 1 and 3. Na and K salts are present, [Na + K] being constant and equal for all compartments. Membranes are not permeable for anions. Na and K permeabilities are chosen according to Ussing's model of frog skin epithelium: membrane (o) (outside) is predominantly Na- and membrane (i) predominantly K-permeable. When starting with high $[K]_2$ and low $[K]_1$ and $[K]_3$, constant inward current is expected to raise $[Na]_2$, thereby increasing resistance and potential of membrane (i). The potential increase alters the Na and K current through membrane (i) according to concentrations and permeability of these ions. The system reaches its steady-state during current flow mainly by alteration of fluxes through the membrane facing the cathode. Generally, $(dK/dt)_2$ is simply $(M_o^K - M_i^K)/d$ where d is the distance between the membranes, but the fluxes M_o and M_i are transcendental functions of concentrations and potentials. Numerical solutions for this system were obtained using a digital computer.

Observations: (1) When current is flowing, the system can show accumulation of a species of cation in 2, on the anodal side of a cathode facing membrane which is predominantly permeable for this species. (2) When the steady state is not yet reached, change of current direction need not reverse the concentration change in 2: when initial concentrations in 2 were extreme, the change in 2 can continue in its former direction, although aiming at a different steady-state value. (3) For a given direction of current flow, the same steady-state concentration in 2 is reached either from above or below (depending on the initial concentration in 2), but not necessarily with the same time course.

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Induced orientation of the growth of malignant cells in vitro. Ruth Johnsson and Andrew Hegyeli.

The partial isolation and chemical characterization of an agent derived from human urine was described. It has been tentatively named directin, because of the way it which it can be identified. It induces directional growth of malignant cells *in vitro* under specified conditions in a system of cells grown on a coverslip in Leighton tubes. The cells become bipolar and arrange themselves in rows, usually parallel to each other and to the long axis of the coverslip on which they are grown.

The urine was concentrated, centrifuged, and the precipitate collected. The precipitate was washed with distilled water at acidic pH. The acidic extract was precipitated with six volumes of methanol, filtered, and the filtrate evaporated to dryness. The dry extract was dissolved in distilled water at pH 2 and passed through Sephadex G-25 gel. The fraction containing direction was chromatographed in butanol-acetic acid-water 4:1:5, and the directin eluted from the 0.15 to 0.25 R_f region. This region gave positive ninhydrin and negative 2-4-dinitrophenylhydrazine reaction. Trypsin, lipase and amylase do not destroy directin. Optimum activity occurs at pH 7.2. It is not destroyed by 56° C. for 30 minutes but partially inactivated at 80° for 10 minutes. Directin can be dialyzed, indicating a molecular weight less than 1000. The extract can be lyophilized. It is stable at −20° C. for 6 weeks.

Possible mechanisms of action were discussed. Three malignant cell lines examined to date respond to directin, whereas five normal ones do not. Examination of living and stained cultures through the polarizing microscope showed long intracellular fibers, but no intercellular ones. Pretreatment of the cultures with DNase did not change the activity. There was no microscopically visible structure to the glass but we were able to determine experimentally that the actual orientation of the treated cells is given by the submicroscopic structure of the glass substrate.

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A cryoprotein in the ocular lens. SIDNEY LERMAN AND SEYMOUR ZIGMAN.

The cold cataract phenomenon observed in the lenses of young animals is due to a specific protein fraction which decreases in concentration as the lens ages. Separation of the cold-soluble and cold-precipitable protein fractions of young lenses using DEAE-cellulose chromatography revealed that the γ-crystallin is the major component of both fractions. The cold-precipitable fraction contained approximately 65% γ-crystallin, whereas the cold-soluble fraction contained 40%. Only the γ-crystallin behaved as a cryoprotein and its ability to precipitate in the cold could be influenced by the following factors. A temperature below 10° C. and a concentration above 3 mg. per ml. must be maintained. Maximum precipitation occurs at pH 6.7–6.8, and 0.25 M urea inhibits the phenomenon. Other lens proteins in solution inhibit cold precipitation when their concentration exceeds that of γ-crystallin. The amino acid composition of γ-crystallin shows that it is a basic protein containing a relatively large number of amino acid residues with apolar side-chains.

The hypothesis that exposed hydrophobic groups are responsible for the tendency of γ-crystallin to precipitate in the cold is consistent with the observation that low concentrations of urea (0.25 M) prevent this phenomenon. It is possible that the α- and β-crystallins tend to keep the γ-crystallin in solution in the normal lens by means of hydrophobic bonding. If either α- and/or β-crystallin are present in a concentration above that of γ-crystallin (as in older animals) the cold cataract phenomenon is no longer observed. Hence this phenomenon can only be demonstrated in the lenses of young animals in which γ-crystallin constitutes the major portion of the soluble lens proteins. The concentration of γ-crystallin in rat lens proteins decreases from a level of 65% in the young (three-week-old) animal to 13% in the 11-month-old rat.

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The role of microtubules in the formation and maintenance of the axopodia of Actinosphaerium nucleofilum; a pressure analysis. LEWIS G. TILNEY AND DOUGLAS MARSLAND.

Electron microscopic preparations were made from specimens of *Actinosphaerium nucleofilum* fixed in glutaraldehyde before, during and after exposure to high pressures (4000–8000 psi).

A study of this material showed that although other organelles were relatively stable, the microtubular elements of the axopodia and cytosome became unstable under pressure. Rapidly they displayed disintegration and such disintegration was correlated with the beading and retraction of the axopodia in the pressurized specimens. Moreover, after the release of pressure, a reappearance of microtubules occurred as soon as or sooner than the re-extension of the axopodia was observed.

The rate of disintegration increased as the pressure was raised. At 4000 psi few if any tubules remained after 10 minutes, whereas at 6000 and 8000 psi the disintegration was much more rapid. Some adaptational reorganization of the microtubules and axopodia occurred while relatively low pressures were maintained. This was accompanied by an actual elongation of the axopodia in specimens maintained for 20 minutes at 4000 psi, but was confined to knob-like axopodial remnants in animals kept at 6000 psi. No regeneration of tubules or axopodia occurred at 8000 psi. The presence of fibrils and a spongy material in pressurized animals suggests that these may be microtubular disintegration derivatives. This morphological evidence tends to confirm the hypothesis that microtubules play an important role in the maintenance of form-stability of axopodia and also in the active processes of axopodial re-extension after retraction.

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The effects of actinomycin D on RNA synthesis in the brackish-water ciliate, Tracheloraphis sp. Reuben Torch.

The lack of detectable macronuclear DNA in *Tracheloraphis* sp. and the organism's capacity for regeneration in the presence of actinomycin D suggest the possibility of information encoding by nuclear and/or cytoplasmic RNA.

Autoradiographs were made of organisms subjected to solutions containing a combination of uridine-H^3 (10 μC./ml.) and variable concentrations of actinomycin D (0, 24, 45, 82 μg./ml.) for variable periods of time (6, 12, 24, 48 hours).

In the absence of actinomycin, the isotope first appears in the macronuclei, and then later in the cytoplasm. As exposure to the isotope is lengthened, cytoplasmic labeling becomes intense, and after 48 hours the entire organism is heavily labeled. Pulse labeling experiments indicate that migration of nuclear material contributes to the cytoplasmic label. In general, the label is sensitive to RNase, but after 48 hours' exposure, a significant amount of label is resistant to RNase, DNase, and a combination of both enzymes.

In the presence of high concentrations of actinomycin, macronuclear labeling is completely inhibited. Nevertheless, cytoplasmic incorporation of uridine-H^3 continues, albeit at a reduced rate. Grain counts indicate that cytoplasmic incorporation is linear over a 48-hour period, even at the highest concentration of actinomycin tested.

If, in *Tracheloraphis*, actinomycin acts by terminating DNA-primed RNA synthesis as reported in other organisms, the results indicate that the organism is capable of DNA-independent cytoplasmic RNA synthesis. It is planned to investigate the site of this activity by means of electron microscopy-autoradiography.

This investigation was supported by research grant GM 11252 from the National Institute of General Medical Sciences, Public Health Service.

Statistical properties of photon emission in bioluminescence. W. A. Hagins, Frank E. Hanson and John B. Buck.

When light is emitted by a black body and the emerging photons are absorbed by a photocathode, the distribution of the resulting photoelectrons in time is approximately Poissonian and the photocurrent shows fluctuations predicted by the theory of the simple shot effect. But if the light source scintillates, bunching of photoelectrons will be observed, and these bunches will increase the fluctuations in the photocurrent above the level characteristic of black body radiation. The steady glows produced by luminous bacteria and by firefly light organs excited by ethyl acetate have been examined for scintillations by studying the noise power spectrum of the output of a photomultiplier which observes a small group of luminescing cells. No scintillations lasting less than 15 milliseconds have so far been found, a 40-photon burst being about the lower limit of detection. This result rules out certain types of processes which might limit the rate of light emission in a steady glow, and this may have bearing on the structure of bioluminescence control mechanisms in general.

Microsources of luminescence in Noctiluca. Roger Eckert, George T. Reynolds and Richard Chaffee.

The luminescence of *Noctiluca* has long been known to arise from numerous minute sources in the thin peripheral layer of cytoplasm which surrounds the hydrostatic vacuole. Hence, the macroflash emitted by the cell is the sum of many microflashes. The nature and behavior of the luminescent microsources were investigated with the aid of both image intensification and microphotometry. The peripheral cytoplasmic layer contains many strongly phase-retarding structures having diameters of 0.5–1.5 μ. By matching photographs (obtained with an image intensifier) of the light emitted by microsources with phase photomicrographs of the same microscopic fields, it was seen that light emission is closely associated with about 15% of the phase-retarding structures. The microflash intensities photographed in a field range over at least one order of magnitude.

In a series of excitations the macroflashes exhibit changes in magnitude dependent on certain temporal parameters. These graded changes in macroflash magnitudes are the result of parallel graded changes in microflash intensities. Sequential photographs of the microflashes occurring in a given microscope field of 20 μ diameter show that all the sources flash in an all-or-none fashion in response to adequate stimulation. When changes in flash intensity occur, all microsources in the field undergo approximately proportionate changes in their luminous output.

Photometric recordings from single microsources show that the microflash has a time-course similar to that of the macroflash. Furthermore, luminescent emission is limited to those phase-retarding inclusions which fluoresce upon excitation with ultraviolet light.

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Light-initiated bioluminescence. J. W. HASTINGS AND Q. H. GIBSON.

Bioluminescence has been viewed as a special case of chemiluminescence involving a reaction mechanism in which the energy from an exergonic chemical reaction is converted to light energy. In recent experiments, carried out with enzyme purified from extracts of the marine bacterium, *Photobacterium fischeri*, it has been found the light energy may in fact be utilized to initiate bioluminescence. This is, of course, not simply the fluorescence or phosphorescence of the emitting species, since the photo-induced emission has a long lifetime, corresponding exactly to that of the chemically-induced bioluminescence—namely from 5 to 10 seconds. The photo-induced luminescence also has the same spectral distribution as the chemically-induced system, and it is similar with regard to its aldehyde-dependence. The excitation spectrum shows a maximum in the region of protein absorption at about 280 mμ, with no evidence of a flavin involvement. This—as well as the lack of flavin stimulation—makes it unlikely that the light-induced reaction proceeds *via* the trivial pathway involving photochemical reduction of flavin. Rather the results give evidence that light populates a long-lived excited state which occurs normally as an intermediate in the chemically-initiated luminescence. Oxygen is required for the light-induced reaction and apparently serves to convert a relatively short-lived (one second) species to the characteristic longer one.

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AUGUST 17, 1965

Studies on ascidian metamorphosis: Birefringence in tail epidermis. JAY LASH AND J. ROUTT REIGART.

Tail resorption in ascidian tadpole larvae is one of the most astonishing events in embryology. Within the short space of 10 minutes, the tadpole tail is resorbed into the head region, heralding the onset of metamorphosis into the adult ascidian. The mechanics of this process had eluded biologists for many years until R. A. Cloney showed with time-lapse photography that the tissue most actively involved with resorption is the epidermis. Analyses of time-lapse movies strongly suggest that the epidermis, after separating from the underlying muscle, undergoes contraction. As a result of this contraction, the tail tissues are pushed toward the head region.

Tadpoles of *Amaroucium* and *Perophora* were examined for birefringence during tail resorption. Fixed and living specimens showed a definite birefringence in the epidermis of the tail, in the subepidermal region of the head, and in the tail musculature. Strongly birefringent granules were also observed in the notochordal cells. Approximate matching of refractive indices indicated that this birefringence is intrinsic to the structure of the material. The birefringence in the tail epidermis may correspond to the fibrous material seen in electron micrographs (Cloney). The birefringence in the subepidermal region of the head corresponds to the region of the mantle wall musculature. These observations support the idea of a contractile tail epidermis in the ascidian tadpole.

Aided by a grant from the National Institutes of Health, HD 00380.

Pressure-induced enhancement of the anti-mitotic effects of colchicine in the cleaving eggs of Lytechinus variegatus. DOUGLAS MARSLAND.

The available evidence indicates that colchicine and high hydrostatic pressure block mitosis by exerting a weakening, or solational, effect upon the gel structure of the mitotic spindle. Consequently it becomes of interest to ascertain whether these two anti-mitotic agencies may have synergistic effects upon mitotic function.

Low concentrations (below 0.00005 *M*) of colchicine and low intensities (below 2800 psi) of pressure, applied separately to early prophase eggs, produced no measurable inhibition of the first cleavage division (at 20° C.). In combination, however, these subliminal treatments produced marked inhibition. Complete blockage, in fact, was observed: at 2300 psi for eggs in 0.00004 *M* colchicine; at 2500 psi for eggs in 0.00003 *M;* and at 2800 psi for eggs in 0.00002 *M* solutions.

The curves obtained by plotting percentage inhibition as a function of pressure for each of the colchicine concentrations specified above were approximately parallel to one another; but each increment in the colchicine concentration shifted the curve about 300 psi higher along the (pressure) abscissa.

In short, a distinct synergism was found in reference to the antimitotic effects of colchicine and pressure. This result is quite opposite to that reported previously in regard to the actions of heavy water and high pressure.

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Identification and isolation of the mitotic apparatus protein. R. E. KANE.

Mitotic apparatuses (MA) isolated from metaphase sea urchin eggs in 12% hexylene glycol at pH 6.4 can be rapidly dissolved in 0.6 *M* KCl. More than one-half of the total protein of the MA is soluble under these conditions and this soluble material consists primarily of the protein of the filaments or microtubules, since the structural organization of the mitotic apparatus can be seen to disintegrate in phase contrast, leaving only dispersed granular material which has been found by electron microscopy to contain no filaments. The amount of protein is in agreement with that calculated to be present in the microtubules from measurements of electron micrographs of isolated MA. Analytical ultracentrifugation of the KCl-soluble material shows it to consist primarily of one homogeneous component with a sedimentation coefficient of 22 Svedbergs. The properties of this component have been studied in detail.

A component of similar physical properties and amino acid composition can be identified in extracts of unfertilized eggs, where it forms approximately 10% of the total cell protein. The isolated MA contains only a small fraction of the total 22S present in the cell and a large amount of this protein can be demonstrated in the soluble supernatant from a mitotic apparatus isolation. The large excess of this protein in the cytoplasm presumably acts as a pool of material which can be mobilized by the cell to form the mitotic apparatus and other microtubular structures. The availability of such a pool of 22S protein in the cell is indicated by the rapid and reversible increases in the number of filaments in the MA which have been observed in other experiments.

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Characterization of the mitotic apparatus protein and its subunits. R. E. STEPHENS.

The major structural protein of the mitotic apparatus has been obtained from spindle isolates, whole egg extracts, and acetone powder from eggs of *Strongylocentrotus droebachiensis, S. purpuratus,* and *Arbacia punctulata.* The material is freely soluble in high salt (0.3 to 0.6 *M* KCl) and distilled water at neutral pH, but shows lesser solubility at 0.1 ionic strength. The protein is free of nucleotide, lipid, and ATPase activity.

Proteins from these species, essentially identical in composition, have characteristically high glutamic and aspartic acid content (25% of total). The purified protein has a sedimentation rate of 22-23S, a diffusion constant of 2.4-2.5D, an intrinsic viscosity of 5.7, a partial specific volume of 0.74 and a molecular weight of 840,000 to 880,000. At pH 11 or higher, a 6.7S subunit of molecular weight 240,000 is obtained. In 8 *M* urea or 4 *M* guanidine·HCl, a 5.3S subunit of molecular weight 110,000 results; addition of 2% mercaptoethanol reduces the sedimentation rate to 2.6S with no decrease in molecular weight. Thus, the 22S particle is formed from 8 subunits (5.3S) which are internally disulfide-linked; mercaptoethanol treatment of these subunits yields a random coil (2.6S).

When the 6.7S or 5.3S subunits are restored, through dialysis, to 0.6 *M* KCl at pH 7.5, the 22S particle is re-formed, accompanied by 13S material, the amount of which is dependent upon length of urea or high pH treatment. The reduced 2.6S subunit cannot be reassociated to the 22S protein. Treatment of the 6.7S subunit with 0.05 *M* Salyrgan, with subsequent dialysis into 0.6 *M* KCl, results in the production of an 11S particle, not reassociable to the 22S protein. These subunit relationships explain most of the discordant literature concerning the character of the protein constituent of the mitotic apparatus.

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GENERAL SCIENTIFIC MEETINGS

AUGUST 23–26, 1965

Abstracts in this section are arranged alphabetically by authors. Author and subject references will be found also in the regular volume index, appearing in the December issue.

Cilia development and associated protein synthesis in the sea urchin embryo.

WALTER AUCLAIR AND DONALD M. MEISMER.

The first specialized structures that appear during early sea urchin development are cilia during blastula. In a study of embryos whose fertilization membranes have been removed, cilia first appear 5–6 hours after fertilization at a length of 5 μ. It is possible to anticipate the appearance of cilia by a gradual change of shape of the embryo from a spherical to an ovoid form.

At one end of the major axis the cilia grow rapidly, 15 μ/hr., until they reach lengths of 45–55 μ, forming the apical tuft region. At the opposite end no cilia develop. This is the region that invaginates, and the cells are of micromere origin. Disaggregation of the embryos with protease-EDTA in Ca-Mg-free medium confirmed this observation. The only non-ciliated cells were small and few in number. Intermediate between these two extremes are blastomeres making the majority of the embryo that have cilia 7.5–20 μ long, following a rough size gradient from the micromere to apical tuft regions. Cilia thus may be considered as being associated only with presumptive ectodermal tissue.

Utilizing a method developed for the isolation of cilia from blastulae, the process of ciliary protein synthesis has been followed through development with C^{14}-glutamic acid and C^{14}-leucine. There is a continued incorporation and accumulation of the labeled amino acids until the fifth hour of development. Dactinomycin (25 μg./ml.) represses this uptake into ciliary protein, but cilia formation still occurs. It thus appears that newly synthesized ciliary protein is not essential for cilia formation to occur.

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Effects of salinity on the oxygen consumption of isolated gills of and on the oxygen consumption and electric potential of intact Fundulus heteroclitus.

JOHN M. AUGENFELD.

The oxygen consumption of *Fundulus heteroclitus,* a euryhaline teleost, was measured in continuously flowing fresh, 40% sea, and 100% sea water, using an oxygen electrode. In fresh water the fish used 0.157 ± .008 ml. O_2/gm. wet wt./hr. and in sea water 0.198 ± .012 ml./gm./hr. ($P < .01$). In 40% sea water they used 0.169 ± .013 ml./gm./hr. Previous acclimation to fresh or sea water for at least two days had no significant effect.

The oxygen consumption of isolated gills was measured by Warburg manometry at 23°, 30°, and 37° C., in isosmotic Ringer's solution and in salt solutions at sea water and fresh water concentrations. At comparable temperatures the Q_{O_2} of the gills was two to three times that of the whole fish. In isosmotic Ringer's, gills of sea-water-acclimated fish used more oxygen than those of fresh-water-acclimated fish at all temperatures, the differences ranging between 10% and 40%. Gill Q_{O_2}s were 5–40% higher in isosmotic Ringer's than in the other media.

The potential difference between a fine 3 M KCl-agar electrode inserted under the skin and a similar electrode in the medium was measured with an electrometer voltmeter. The Nernst equation predicts a potential of +18 mv. of the fish with respect to fresh water and of —6 mv. to sea water, if no active transport occurs. Acclimated fish are *ca.* 15 mv. negative in fresh water, indicating that they actively transport chloride into their body fluids. In 4 mM sodium sulfate their potential drops to —2 mv., but rises to the original level when the fish were returned to fresh water. Sea-water-acclimated fish are *ca.* 3 mv. negative in fresh water, indicating that they lose chloride faster than fresh-water-acclimated fish do. Both sea- and fresh-water-acclimated fish are *ca.* 15 mv. negative to sea water, indicating that they actively excrete sodium. Ouabain and eserine, which are known to interfere with sodium transport, reduce these potentials.

Work done during the Comparative Physiology Program at the Marine Biological Laboratory, supported by the National Institutes of Health, TH 5T 1 GM 1030-03.

Behavioral studies on the commensal amphipod crustacean, Listriella clymenellae Mills. RUTHANNE BATCHELLER AND ERIC L. MILLS.

Listriella clymenellae, a small amphipod crustacean, is known in the Cape Cod area as a commensal in the tubes of the maldanid polychaete, *Clymenella torquata* Leidy. Although the morphology of the species has been studied, only a few rudimentary observations on its biology have been published previously.

Locomotion on level surfaces is by the action of pereiopods 1-4. The large spinose pereiopods 3-5 allow purchase on the inner tube wall. Swimming is by the current of the pleopods and flexion of the urosome. *Listriella* can also burrow actively. It can live apart from the host for long periods and probably moves often from one tube to another.

There is a strong negative reaction to light, which partly accounts for *Listriella*'s tendency to enter tubes. Normally, random movements bring the animals into contact with *Clymenella* tubes. Laboratory experiments suggest that the initial response to the tube is largely tactile. After an exploration of the tube surface, *Listriella* enters the tube. Substances produced by the polychaete are important in keeping the amphipod in the tube. Experiments suggest that mucus is the important factor. *Listriella* responds positively to the mucus of *Pectinaria gouldi* (Verrill) as well as to that of *Clymenella*, so the response is largely non-specific. The association with *Clymenella* is probably maintained because its respiratory currents and feeding methods are compatible with those of the amphipod.

Food is gathered by the large gnathopods and is compacted by them and passed to the mouthparts. It is likely that food is also carried to the head region by the strong antero-posterior pleopod current.

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An RNA polymerase system from Arbacia. Extraction and properties. DANIEL M. BERKOWITZ, ELLEN M. BERKOWITZ AND WALTER TROLL.

The synthesis of RNA during the early development of an organism is of obvious interest, since RNA is a fundamental component in the expression of genetic information. We have isolated and purified a highly active RNA polymerase from the unfertilized eggs and the blastulae of *Arbacia punctulata*. This enzyme is unusual in the following ways: (1) it is insensitive to a DNA primer and to inhibition by actinomycin D; (2) it has a specific activity

7000 to 50,000 times as great as RNA polymerases isolated from other animal tissues, an activity in the range of those isolated from bacteria. The egg and the blastula preparations differ from each other in the following ways: (1) the egg enzyme is more sensitive to polyribonucleotide primers; (2) the blastula enzyme has a specific activity 7 times that of the egg enzyme; (3) the omission of GTP from the reaction mixture causes a decrease in the activity of the blastula enzyme, but an increase in that of the egg enzyme. Both enzyme preparations, on the other hand, synthesized poly A to a greater extent than RNA. Essentially, the method of isolation consisted of homogenization, ultracentrifugation, precipitation of the nucleic acids by protamine, extraction of the enzyme with sodium succinate, precipitation by ammonium sulfate, and Sephadex chromatography. The assay mixture consisted of Tris buffer, mercaptoethanol, magnesium and manganese ions, the four ribosetriphosphates, one of which was tritiated, and the enzyme. The assay was carried out at 30° C. for 20 minutes, and was terminated by the addition of cold perchloric acid. Acid-insoluble material was collected on Millipore filters, washed, dried, and counted in a liquid scintillation counter. We believe that the enzyme prepared by this method may represent the actinomycin-D-resistant RNA-synthesizing activity described by Gross and co-workers.

The effect of puromycin and actinomycin on cell division, protein synthesis and malate dehydrogenase in sea urchin embryos. R. B. BILLIAR, J. B. BILLIAR, C. A. VILLEE AND L. ZELEWSKI.

Sea urchin embryos were maintained in sea water for varying times in the presence or absence of puromycin and actinomycin D. Alanine-2-^{14}C was added and the embryos were shaken gently by hand for 20 minutes, then collected by centrifugation and homogenized in 0.1 M citrate buffer, pH 6. Portions of the homogenate were removed for ^{14}C-protein analysis and the remainder was centrifuged at about 8000 g for 30 minutes. The supernatant fraction was analyzed spectrophotometrically for L-malate dehydrogenase (L-MDH) activity and the pattern of L-MDH isozymes was determined by disc electrophoresis on polyacrylamide gel. Puromycin, 20 μg./ml., added three hours before fertilization, inhibited cell division but did not prevent fertilization. Puromycin added 12 or 15 hours after fertilization prevented further embryonic development but did not prevent active swimming of the embryos. Puromycin added three hours before fertilization or at 12 or 15 hours after fertilization inhibited protein synthesis, as measured by a 20-minute pulse with alanine-2-^{14}C, at 4, 18 and 22 hours, respectively. Actinomycin, 20 μg./ml., slowed the rate of cell division but the embryos formed cilia and some hatched. Development in the presence of 10 μg./ml. of actinomycin appeared to be normal to hatching. With 10 μg./ml. actinomycin added three hours before fertilization the rate of protein synthesis at four hours was equal to that of controls but at 16 hours was decreased to 25% of controls even though the embryos had hatched. Protein synthesis in embryos grown four hours in 20 μg./ml. actinomycin was 80% of controls. The total L-MDH activity of the embryos was unaffected by any of the treatments. No marked change in the pattern of L-MDH isozymes was produced by any of the treatments. Thus L-MDH appears to be a rather stable protein during early embryonic development.

The reproductive cycles of Arbacia punctulata and Strongylocentrotus dröbachiensis. RICHARD A. BOOLOOTIAN AND VILIA TURNER.

Strongylocentrotus dröbachiensis (north of Cape Cod) and *Arbacia punctulata* (south of Cape Cod) were collected monthly and bimonthly, respectively, from July, 1964, to August, 1965. Their gonadal indices (G.I.) were determined (ratio of gonad volume to total wet weight ×100). The *S. dröbachiensis* breeding season was November to April, with a peak G.I. in January. Spawning occurred from late January to mid-April. Following spawnout ripe gametes could be obtained as late as June. The G.I. never went below 6.5%; hence the gonads remained relatively large during the rest period. The temperature range within which *S. dröbachiensis* developed from fertilization to metamorphosis was 3°–10° C. with an optimum of 4°–7° C.

A. punctulata exhibited two annual breeding seasons, during which ripe gametes could be obtained. The summer breeding period from June to August is well-known. A winter breeding period, from early December to January, had never been suspected prior to our study. When the study began the G.I. was 5.2% and dropped to 1.5% in August. Between August and the first week of December the G.I. oscillated, resulting in three minor peaks in September, October, and November; however, gametes were not present. Within the first week of December the G.I. reached 13.4%, two weeks later dropped precipitously to 3.5%, one week later rose sharply again to 12.7%, and then in two weeks dropped to a low of 2.3%. From mid-January to late May there were small peaks following which the G.I. rose to a high of 9.4% in July and dropped in August to 2.3%.

Gametes obtained from *A. punctulata* during the winter breeding period were fertilizable at all temperatures but failed to develop beyond the second or third cleavage stage at the ambient sea water temperature (1–2° C.). However, cultures taken to 20° C. ± 1.5° developed into normal plutei.

The adaptive significance of the winter breeding season is not understood, particularly since *A. punctulata* becomes inactive during winter as growth and feeding virtually stop.

Supported by NSF GB 1042 to R. A. B.

Variations in spacing between axon and Schwann membranes induced in lobster nerve fibers by currents and fluxes. PHILIP W. BRANDT, ALAN R. FREEMAN, JOHN P. REUBEN AND HARRY GRUNDFEST.

The axon and its surrounding Schwann cells are bounded by unit membranes about 60 Å thick, separated by a periaxolemmal space uniformly about 100 Å wide. Adjoining Schwann cells are separated by a convoluted cleft also about 100 Å wide, which connects the periaxolemmal space with the exterior. Hyperpolarizing currents (2×10^{-7} amp., lasting 2 minutes or more) applied with a KCl-filled intracellular electrode produce a marked change in appearance, observed with phase microscopy. In electron micrographs the periaxolemmal space shows focal expansions or vesiculations which become larger and more numerous with increasing duration and intensity of the inward current. Outward (depolarizing) currents produce only occasional small vesiculations. Vesiculations have not been seen in control preparations, nor when inward currents are passed with a microelectrode containing propionate as the anion. These findings suggest that the vesiculations result from temporary accumulation in the periaxolemmal space of Na migrating toward the axon and of Cl leaving the axon during inward flow of current. Various degrees of vesiculation are also produced under other conditions in which it is likely that movements of ions across the membrane take place and cause local ionic and/or osmotic inhomogeneity in the periaxolemmal space. Severe vesiculation occurs on returning a fiber to the control saline from hyperosmotic KCl or glycerol solutions. Smaller and fewer vesiculations develop on placing fibers into hyperosmotic or hyposmotic NaCl salines. Still less vesiculation occurs on transfer of the fiber from a Cl to an isosmotic propionate saline; prolonged repetitive stimulation; or exposure to a hyperosmotic propionate saline. Technical Note: Isolated axons fixed in osmic acid or prefixed in 5% glutaraldehyde present flattened cross-sections. Pre-exposure to 20% hemoglobin or 0.5% glutaraldehyde added to control saline avoids this distortion.

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Isolation of nuclei from red algae. A. E. BROOKS, PETER C. MALONEY, REGINA L. KORNBLITH, VICKI L. GIORDANO AND MAIMON NASATIR.

Nuclei have been isolated from several multinucleated genera of red algae including: *Polysiphonia urceolata, P. denudata, P. flexicaulis* and *Griffithsia globulifera*. The progress of the isolation procedure was followed microscopically by staining with aceto-lactic orcein, and all steps of the isolation were carried out at 0° C.

Clean fresh algae were homogenized for 5 minutes at approximately 5000 rpm in a Serval Highspeed Omni-mixer. The cell disruption was effected in a sucrose grinding solution, pH 7.4, containing 0.003 *M* $CaCl_2$ and 0.003 *M* Tris. Sucrose concentration could be varied between 0.75 *M* (isotonic) and 1.5 *M* without appreciably altering the yield of nuclei. In hypertonic solution nuclei were thought to be more resistant to mechanical breakage. The homogenate was then filtered through flannelette to remove intact cells, cell wall fragments and associated gelatinous material. Centrifugation of the filtrate at 3800 *g* resulted in a pellet enriched for nuclei. After washing in grinding solution, nuclei could be stored at 0° C. in ethanol.

An average of 100 g. fresh weight of algae was required to give a gram nuclear pellet. Good yields of DNA could be extracted from nuclear pellets stored two weeks, whereas extractable DNA was reduced in samples stored for longer periods of time. The DNA prepared from isolated nuclei contained less contaminating polysaccharide material than DNA prepared from whole cells.

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Effect of cold temperature acclimation and size on the respiration of ascidians. ALBERT J. BURKY AND A. FARMANFARMAIAN.

Cold temperature acclimation and body size have been studied in relation to metabolism in many groups of animals, but in this respect there is little information available for the ascidians. Using the direct method of Warburg, oxygen consumption was studied in relation to body size in *Ciona intestinalis, Amaroucium constellatum* and *Botryllus schlosseri.* Temperature acclimation was investigated in *C. intestinalis.*

For animals collected from the field the average rate of oxygen consumption in ml. O_2/hr./kg. wet is 108 for *B. schlosseri;* 33 for *A. constellatum;* and 9.9 for *C. intestinalis* at 20° C. (average ambient temperature early in August). A size index was arbitrarily defined as the product of maximum length and the average external diameter of the animal or zooid. When the size index of *B. schlosseri* zooid, which is the smallest of the three species, is taken as 1, *A. constellatum* has a size index of 8.9 and *C. intestinalis* a size index of 1510. The respiratory rate has an inverse relation to size as it has been reported for other animals.

Ciona intestinalis acclimated to 6° C. for 34 days showed an increase in the metabolic rate over the controls treated similarly at 18° C. This increase was observed at all temperatures of measurement. The rates of oxygen consumption in ml. O_2/hr./kg. dry for acclimated animals at measurement temperatures of 5°, 10°, 15°, 20° and 25° C. are 127, 202, 445, 1019 and 777, respectively, while those for controls are 86, 139, 234, 377 and 535, respectively. Animals acclimated at 11° C. were generally similar to the controls in their metabolic pattern. This is probably due to the facts that the acclimation temperature was too close to the control temperature and the acclimation period was too short. These data indicate partial compensation with a clear translation pattern. Since the acclimation period was short and few animals were used, the rotation pattern could not be precisely established, but a clockwise pattern seems to be indicated.

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Reorganization of spindle components following UV microirradiation. RICHARD D. CAMPBELL AND SHINYA INOUÉ.

To investigate the interactions between centriolar function and birefringence of metaphase spindle fibers, lesions were made in the metaphase-arrested spindles of *Pectinaria gouldi* oocytes, using a microbeam of ultraviolet light. Birefringence could be locally abolished in spindle regions between the two poles. The missing birefringence reappeared during the minutes following irradiation. The unirradiated astral regions, appearing as minute birefringent crosses under the polarizing microscope, remained unaffected in these control experiments.

When the microbeam was directed at one spindle pole, in the region of the centriole, two types of lesions were observed. Sometimes only the astral birefringence was lost and subsequently regenerated. Frequently, however, local polar irradiation led to loss of the entire half-spindle. This indicates that the organization of a half-spindle is partially dependent upon the intactness of the centriolar region.

The lost half-spindle was reformed by reorganization of the residual spindle material, rather than by reappearance of birefringence in the original position. The fibers adjacent to the lost birefringence converged to form a new pole, where astral birefringence concomitantly appeared. Sometimes small asters formed at the tips of prominent residual fibers before convergence, and later merged.

The regenerated asters could represent *de novo* formation of centrioles following their destruction. More probably, centrioles or their fragments are pulled on the residual spindle fibers to new positions, where they proceed to act as reorganizing centers.

These experiments were carried out in 40% deuterated sea water to increase the size and birefringence of the spindle. The cells were flattened under a coverglass to reduce cytoplasmic absorption of ultraviolet light.

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A thermodynamic analysis of the effect of D_2O and H_2O on the mitotic spindle.

ROBERT M. CAROLAN, HIDEMI SATO AND SHINYA INOUÉ.

Thermodynamic analyses have been made on the formative reaction of the mitotic spindle fibers of *Pectinaria gouldi* oocytes in H_2O and 45% D_2O sea water. The concentration of oriented material in the spindle is measured with the polarizing microscope as retardation induced by spindle fiber birefringence. The retardation disappears around 8° C., peaks near 21° C., then diminishes at higher temperatures. D_2O extends the retardation to a lower temperature and increases it almost two-fold at its peak.

Arrhenius plots ($\log B/(A_o - B)$ versus 1/temperature, where B is spindle retardation and A_o the asymptote B approaches) showed: linear functions between 8° and 21°; the reaction is endothermic with a high $+\Delta H$, high $+\Delta S$ and low ΔF; ΔH and ΔS are about double the values calculated for *Chaetopterus pergamentaceus;* ΔF is 0 at about 13° C. *versus* 19° C. for *Chaetopterus.* Here the ratio of free to polymerized monomer concentrations should be one.

The data support the hypothesis that retardation reflects the reversible association of protein monomers into linearly aggregated polymers in a first-order temperature-driven reaction and the hypothesis that in polymerization, the monomers release water which is either bound to it or structured about its hydrophobic groups. These thermodynamics resemble those for the polymerization of TMV protein and of G–F transformation of G-ADP-actin.

Comparing the measurements made in H_2O and 45% D_2O: ΔH in $H_2O = 58.4 \pm 9.8$ kcal and in $D_2O = 44.1 \pm 4.8$ kcal; ΔS in $H_2O = 204 \pm 33$ eu and in $D_2O = 154 \pm 16$ eu. Both differences are significant to the 0.02 level. Assuming that here ΔH and ΔS primarily reflect the randomization of water molecules as they are displaced from the monomer, these values indicate that per monomer polymerized, D_2O becomes less disorganized than H_2O, perhaps because D_2O is itself better associated than H_2O.

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Aspects of ultrastructure and cytochemistry during oogenesis in Artemia salina.

REV. JOSEPH D. CASSIDY, O.P.

Morphological components of developing germ plasm from adult female *Artemia* were studied with the aid of recent improvements in cytological methods and high resolution electron microscopy. Ovarian tissue was dissected, taken through six changes of 5% glutaraldehyde prepared in Millonig's phosphate buffer at pH 7.45, for two hours, and then transferred to a buffer supplemented with 10% sucrose. One-half of this solution was decanted and replaced with 2% OsO_4 for 1.5 hours. The temperature was maintained throughout this Fitzjarrel schedule at 4° C. Electron staining with saturated aqueous uranyl acetate for 1.5 hours was followed by rapid alcoholic dehydration, Maraglas infiltration and polymerization at 52° C. Silver sections were supported on coated grids and examined in the Hitachi 7S and Siemens IB electron microscopes. Alternate 1 μ sections were attached to glass slides with 10% acetone for correlated cytochemical determinations, according to the reactions of Azure B, Feulgen, PA/S, methylene blue, Sudan black, Nile blue A, and basic fuchsin.

Preliminary identification of fine structures in the oocyte indicated the presence of clusters of mitochondria, Golgi zones, lamella, and microvilli at the cortex. Protein, lipid, and carbohydrate yolk was localized. Studies on the trophocyte-oocyte association suggest that the nutritive cells provide a source of RNA, and also that the intact nurse cell nuclei may be engulfed by the maturing oocyte. The possible role of the cortical microvilli in vitellogenesis remains to be assessed.

Evidence against the concept of growth zones in hydroids. SEARS CROWELL, CHARLES R. WYTTENBACH AND ROBERT L. SUDDITH.

Contrary to the widely held assumption that growth in hydroids occurs in localized zones of proliferation are the recent findings of several investigators. Though the observations are scattered, collectively they show that zones which seem to be meristematic are, in fact, merely zones of outthrust resulting from proliferation from behind.

If mitosis is localized, it should be evident histologically. Yet, observations by Lunger (1962), Wyttenbach and Wyttenbach, Thabes and Pasternak and Crowell give no evidence for cell division in stolon or stem tips in *Campanularia.* Similar conclusions have been reported by Hale (1964) in *Clytia* and Overton (1963) in *Cordylophora.* By means of tritiated thymidine localization, Campbell (1965) shows clearly that in *Hydra littoralis* mitosis is widely scattered through the upper column, not restricted to the subhypostomal region.

Vital staining (Nile blue sulfate) of *Campanularia* uprights by Wyttenbach (1965) demonstrates that tissues near the base of the upright move distally at the same rate as terminal elongation occurs. Our similar study of stolon elongation shows that cell proliferation takes place not at the tip but at a distance of as much as 2 to 4 mm. behind it. The findings are consistent with vital staining experiments by Hale and Overton on *Clytia* and *Cordylophora,* respectively.

Finally, Crowell (1961) found that when a young hydranth bud of *Campanularia* is isolated, it differentiates into only the distalmost parts of a hydranth. This is most consistent with the idea that the cells of a hydranth bud move into place from the pedicel below.

Collectively, these data argue strongly against the concept of localized growth zones, a concept which has been in vogue for fifty years (Kühn, 1914). Instead, proliferation occurs in regions distant from the sites of its outward manifestation.

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Effects of hydroxyurea on the early development of Arbacia punctulata. HENRY W. EISENBERG, DINA VAN PRAAG, HERBERT S. ROSENKRANZ AND DAVID SHEMIN.

In bacterial and mammalian cells hydroxyurea is a specific and reversible inhibitor of DNA synthesis. This property of the drug has been shown to be useful in the elucidation of the role of DNA in a number of biological phenomena. The present study was undertaken to ascertain whether hydroxyurea could be useful in studying the control of embryonic development of *Arbacia punctulata.* It was thus found that concentrations of the drug of the order of 10^{-2}–10^{-3} M stopped cleavage at the two- and four-cell stages while lower concentrations (*ca.* 5×10^{-4} M) effectively blocked development at the blastula stage. Evidence of the reversibility of the block was obtained when fertilized eggs were exposed to 10^{-3} M hydroxyurea for two hours and the drug removed by dialysis against sea water.

Metabolic experiments indicated that even when development was stopped at the two-cell stage, the incorporation of C^{14}-leucine into protein was 70% of the control. The effect of hydroxyurea on the incorporation of C^{14}-thymidine (C^{14}-TdR) and C^{14}-uridine (C^{14}-Ur) closely paralleled the ability of the drug to interfere with embryonic development. Thus, when cleavage was blocked at the two-cell stage, the uptake of C^{14}-TdR and C^{14}-Ur was negligible. When differentiation was blocked after blastulation, the incorporation of the two nucleic acid precursors was reduced by approximately 50%. On the other hand, if the hydroxyurea concentration was reduced to 10^{-4} *M* such as to allow the embryos to reach the late gastrula stage, the uptake of C^{14}-TdR proceeded normally while the incorporation of C^{14}-Ur was still inhibited by approximately 40%. Urea at equivalent concentrations was without effect on the development of fertilized eggs nor did it reverse the effect of hydroxyurea.

These results are compatible with an effect of hydroxyurea on the ability of DNA to serve as a template for both DNA and RNA synthesis. Indeed a direct *in vivo* effect of the agent on *Arbacia* DNA could be demonstrated by cesium chloride density gradient centrifugation. Current radioautographic studies will undoubtedly be helpful in elucidating further the mode of action of hydroxyurea on developing embryos.

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Physiological considerations of the controversial echinoid "heart." A. FARMANFARMAIAN.

Recently the aboral terminus of the axial organ situated below the madreporic plate of the sea urchin has been described as a "primitive heart" and its contractions likened to those of a two-chambered heart with auricular and ventricular beats. It has further been claimed that the primitive heart moves coelomic fluid from the perivisceral cavity into and throughout the hemal system of the sea urchin, *Strongylocentrotus purpuratus,* and that a "true circulatory system" has been demonstrated. My published investigations on the transport of oxygen from sea water to the visceral organs and the transport of nutrients from the digestive tract to the other tissues in the same species indicate that the transport of these substances is mainly directly *via* the perivisceral fluid. The hemal system does not have a significant role in the transport of these substances, since the radial hemal vessels and other vessels may be severed without adverse effect on the mentioned transport. A current series of experiments on *Arbacia punctulata* and *Strongylocentrotus dröbachiensis* involved measurement of oxygen consumption of whole animals before and after the madreporic plate, the "heart," and the axial complex were all excised and the wound packed with Bacitracin-impregnated cotton. Each animal served as its own control. The normal oxygen consumption of five *Arbacia* at 20° C. in µl./hr./g. wet was 17.8; 23.4; 26.2; 24.9; 25.2 and after the operation 17.8; 23.8; 26.2; 24.9; and 22.4, respectively, for the same animals. Similarly obtained results for *Strongylocentrotus* were 35.0; 33.4; 35.7; 39.2; 34.7; and those after the operation were 33.6; 31.0; 38.7; 38.1; 33.4, respectively, for the same animals. Under appropriate conditions the animals survive well after the operation and regeneration of the madreporic plate has been observed for *Arbacia* after 20 days. These and other experiments which will be detailed in a later communication indicate that the claimed "heart" has no significance in the immediate transport of oxygen or nutrients and that the structure is essentially physiologically dispensable and may be regenerated like other parts.

Comparison of the hormones controlling the red chromatophores in the fiddler crab, Uca pugilator, and the prawn, Palaemonetes vulgaris. MILTON FINGERMAN, KARLYNN WENGER AND YOSHIHIRO YAMAMOTO.

The fiddler crab and prawn both produce concentrating and dispersing substances for the pigment in their erythrophores. This investigation was undertaken primarily to determine whether the chromatophorotropins that act on the red pigment of one species will cause migration of the red pigment in the other. Sinus glands and central nervous organs from both species were extracted in sea water and injected into both species for assay on dispersed and concentrated red pigment. Most extracts were quite effective both in concentrating and dispersing red pigment in each species. Other extracts had a large concentrating or dispersing effect in one species and little or no effect on the pigment in the corresponding stage in the other species. For example, sinus glands of *Palaemonetes* produced virtually no red pigment dispersion in *Palaemonetes* but had a very large dispersing action on the red pigment of *Uca.* In contrast, the tritocerebral commissure of *Palaemonetes* caused no red pigment dispersion in either species. Analysis of all of the data revealed that although both the prawn and the crab contain dispersing and concentrating substances for the red pigment of both species, the substances that activate crab erythrophores are different from those effective on prawn erythrophores. In other words, each species contains at least two red pigment-dispersing substances and two red pigment-concentrating. Furthermore, the substance from *Palaemonetes* that disperses the red pigment of *Uca* is different from the hormone in the prawn that disperses the melanin of *Uca*. Only when an extract was prepared in a solution of sodium chloride was the red pigment-concentrating effect of the tritocerebral commissure from *Palaemonetes* on isolated chromatophores as large as observed with extracts in sea water. The other cations tested were potassium, lithium, calcium, and magnesium.

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The post-commissure organs of the fiddler crabs, Uca pugilator and Uca pugnax. MILTON FINGERMAN, YOSHIHIRO YAMAMOTO AND KARLYNN WENGER.

The gross anatomy of the post-commissure organs, neurohemal organs near the esophagus, has not been described for any species of fiddler crab. Furthermore, they have not been removed and studied physiologically in any crab. As in other crabs that have been investigated, in both species of *Uca* paired post-commissural nerves arise from the anterior surface of the tritocerebral commissure which lies posterior to the esophagus. However, in *Uca pugilator* the nerves are widely spaced, each orginating close to one of the circumesophageal connectives, but in *Uca pugnax* the roots of the post-commissural nerves lie very close to each other, near the center of the tritocerebral commissure, a condition not previously reported for any other crab. In both fiddler crabs, as in other crabs, each post-commissural nerve passes anterolaterally along the dorsal surface of the *musculus dilatator internus pylorici inferior* to terminate in a network of fibers on the surface of the anterior third of the *ligamentum ventrale capitis.* This network of nerves lies next to a dorsoventral venous channel. After each eyestalk stub of eyestalkless specimens of both species was stimulated by means of a red hot electric cautery for three seconds, melanin-dispersing and red pigment-dispersing hormones were released, presumably from the post-commissure organs. The effect on the melanin was greater than on the red pigment. Extracts containing one-third of both post-commissure organs per dose from *Uca pugilator* were injected into crabs having maximally concentrated black and red pigments and into those whose pigments were maximally dispersed. Dispersion of the black and red pigments occurred, as well as concentration of the red. The responses were comparable to those of crabs administered an extract containing one-third of the circumesophageal connectives per dose.

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Chloride movement and excitation-contraction coupling in isolated crayfish muscle fibers. HORACIO GARCIA, JOHN P. REUBEN, PHILIP W. BRANDT AND HARRY GRUNDFEST.

The role of chloride in excitation-contraction coupling was indicated in earlier studies. Contractions occur merely on reintroducing Cl after a fiber had been kept in a Cl-free medium. In the presence of Cl, contractile activity occurs while the fiber is becoming hyperpolarized, or is repolarizing from previous depolarization. The present work demonstrates further that contraction is not dependent simply upon the reduction of the membrane negativity by some threshold depolarization. The normally graded electrical response of the fiber to brief intracellular stimuli is converted to a propagating spike by adding 10^{-3} gm./ml. procaine to the bathing solution. The spike is greatly prolonged if an impermeant anion is substituted for Cl. Nevertheless, only when Cl is present does the spike evoke a substantial contraction. In fibers not treated with procaine, intracellularly applied outward (depolarizing) currents elicit contractions which are maintained as long as the current is applied, provided Cl is present, but are of brief duration if Cl is absent. Inward (hyperpolarizing) currents also evoke contractile activity, but only when Cl is present. The contractions depend upon the duration as well as the intensity of the applied currents, increasing with the duration for a given intensity of current. The contractile activity is similar to that which occurs when removal of K from the bathing medium results in hyperpolarization or repolarization of the fiber. These data therefore support the hypothesis of Girardier, Reuben, Brandt and Grundfest (*J. Gen. Physiol.*, **47**: 189, 1963) that the agent for excitation-contraction coupling in crayfish muscle fibers is the circulation of current across the plasma membrane and that of the transverse tubular system (TTS). The current is channelled by the anion-permselective characteristic of the TTS component of the cell membrane.

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The distribution of microtubules in primary and secondary mesenchyme in Arbacia. JOHN R. GIBBINS AND LEWIS G. TILNEY.

Pseudopods (filopods) have been shown to be directly involved in morphogenetic movements in sea urchin development. The filopods of the primary mesenchyme cells provide the basis for individual cell movement and the production of skeletal pattern, and those of the secondary mesenchyme are responsible for the extension of the archenteron. Particle translation and waving motions have also been observed in both types of pseudopods and demonstrate a similarity to other cell systems in which microtubules are present. In anticipation of finding microtubules in mesenchymal pseudopods, sea urchin embryos were fixed at several stages during the indentation and extension of the archenteron and examined in the electron microscope.

Microtubules were found in the pseudopods of both primary and secondary mesenchyme cells. Generally, the primary mesenchyme filopods contained more microtubules. In both cases the microtubules frequently occurred in pairs and were surrounded by a zone from which cytoplasmic components were excluded. Vesicles, both coated and smooth, and vacuoles containing yolk spheres were often present in the vicinity of the microtubules.

Experimental work is in progress to determine more specifically the role of the microtubules in pseudopodial function and as a result their role in the determination of morphogenetic pattern.

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Effects of high temperatures on the prosobranch snail, Littorina littorea. ROBERT J. HAMBY AND G. FRAENKEL.

Littorina littorea was exposed, submerged, to constant temperatures ranging from 39–45° C. for one hour and subsequently returned to cool sea water for 24 hours. The effects of these temperatures were measured by the following criteria: (a) behavioral response (ability to climb up the side of their container out of the water), (b) contraction of the columellar muscle in response to single electrical shocks, and (c) observation of the pattern of spontaneous action potentials in nerve tracts, using a suction electrode.

By the behavioral criterion, 50% mortality occurs after exposure to 41° C. for one hour. Death is not instantaneous as animals subjected to 44° C. still respond noticeably to tactile stimulation over 24 hours later. Contraction of the columellar muscle was measured by clamping the snail's shell, hooking a pin attached by a string to a transducer through the operculum, stimulating the muscle, and recording the contraction curves on a Grass polygraph. Normal contractions consisted of a phasic contraction followed by a small tonic and smaller spontaneous contractions. Removal of the ring and pedal ganglia abolished tonic and spontaneous contractions and prolonged the relaxation phase of the phasic contraction. Snails previously subjected to 44° C. exhibited a reduced phasic contraction and enhanced tonic contraction with long relaxation phase. Deganglionation abolished the tonic contraction and reduced the amplitude of the phasic contraction. Weak tonic contractions were also evident in snails exposed to 45° C. Strength-duration curves after exposure to 40° C. were congruent with those of normal animals, whereas those of animals subjected to 41° C. showed a shift toward longer chronaxies. The 44° C. curves were shifted further toward longer chronaxies. The pattern of spontaneous activity of the tentacular nerve was considerably different in the normal and 44° C. snail, with lower amplitude and less frequent spikes resulting from exposure to 44° C. These data suggest that differential injury to components of the nervous system may result from subjection to supranormal temperatures.

Studies on substrate preferences of the sea urchins, Arbacia punctulata and Strongylocentrotus dröbachiensis. BARRY M. HEATFIELD.

The importance of the substratum in influencing local distributional patterns of two species of sea urchins was studied experimentally by analyzing preference responses of 50 animals of each species to substrate particle size, mobility, and texture.

Studies on particle size preferences were made on all combinations of sand (1.0–0.25 mm.), coarse sand (4–5 mm.), fine gravel (8–10 mm.), coarse gravel (18–23 mm.), rock (35–45 mm.), and concrete. Substrate combinations were arranged in a picture-picture frame relationship in trays submerged in sea water. Animals were placed singly on the four, level boundaries between substrates. The number of *A. punctulata* selecting each substrate was directly proportional to particle size. However, no correlation was obtained for *S. dröbachiensis* below particle sizes of 35–45 mm.

Studies of responses to substrate mobility were made on coarse sand and fine gravel, unglued and glued with Fiberglas resin to Plexiglass plates. In all combinations of these substrates, *A. punctulata* preferred glued to unglued substrates, irrespective of particle size. However, no distinction was made between glued and unglued fine gravel. Furthermore, glued coarse sand was preferred over glued fine gravel. A general lack of preference was shown by *S. dröbachiensis* in these experiments.

Studies on texture preferences were made on all combinations of glass, frosted glass and sand glued to a Plexiglass plate. Other combinations between glass and polished and unpolished granite, and glass and Fiberglas cloth were also used. Both species preferred smooth to coarse surfaces, though preferences were more marked in *A. punctulata.*

These species are found on gravel or rock in their respective habitats. The results of the above experiments suggest that their distribution can be explained in part by preferences for larger, more continuous and smoother substrates. This is particularly true for *A. punctulata.*

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Voltage clamp measurements of the Cl-conductance changes in skate electroplaques. BERTIL HILLE, MICHAEL V. L. BENNETT AND HARRY GRUNDFEST.

Electroplaques of *Raja erinacea* were voltage clamped with two low-resistance intracellular microelectrodes. Strictly isopotential conditions could not be maintained in the large cells. However, the technique provided new data on the electrically excitable conductance increase which occurs in rajid electroplaques and which has been ascribed to depolarizing Cl-activation. The resting potential usually was close to −65 mv. For hyperpolarizations to about −140 mv and for depolarizations to about −50 mv the current changed to steady values along a linear I-E relation which indicated cell resistances of 5 to 15 kilohms in different experiments. For larger hyperpolarizations the current fell slowly from a peak value, indicative of the hyperpolarizing inactivation responses that are observed in current clamp measurements. For larger depolarizations the same linear I-E relation was observed only during a brief period after applying the voltage step. The current then increased to a new steady value, indicating an increase in membrane conductance. The increase developed slowly for depolarization to −45 mv ($t_{\frac{1}{2}}$ about 25 msec.), but the rise was faster for larger depolarizations, $t_{\frac{1}{2}}$ attaining a minimum of about 3 msec. at a membrane potential of about −10 mv. The maximum currents increased along an S-shaped curve until the membrane was depolarized to about −25 mv, when the I-E relation again became linear, but with a slope which indicated a 5- to 15-fold conductance increase in different cells. The new slope extrapolated for zero current to a potential about 5 mv positive to the resting potential. The magnitude of the conductance increase was unaffected by tetrodotoxin (5×10^{-7} g./ml.), or by removal of Na and/or Cl from the medium. However, when an impermeant anion (propionate) was substituted for Cl, brief depolarizing stimuli elicited prolonged spikes, independently of the presence or absence of Na. The spike was associated with a conductance increase and voltage clamped cells exhibited an inward flow of current. These data thus confirm the earlier conclusion that the spikes are generated by an efflux of Cl when the electrochemical gradient for this anion is altered so as to permit an inward current during depolarizing Cl-activation.

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The effects of oxidizing, reducing, and sulfhydryl reagents on the resting and action potentials of the internally perfused axon of Loligo pealeii. F. HUNEEUS-COX AND B. H. SMITH.

Cysteine-HCl (400 m*M*, pH 7.3) was used to remove axoplasm from the giant axon of *Loligo pealeii.* After cysteine treatment and prior to the test of the effect of a given compound, a 10-minute perfusion with a 600 m*M* potassium fluoride-potassium glutamate standard medium allowed the axon to reach a steady functional state (resting potential, −50 to −60 mV; action potential, 105–120 mV).

Three oxidizing agents (quinone, chloranil, and hydrogen peroxide) in concentrations of 1–60 m*M* (in standard medium; pH 7.3–7.4; 16–18° C.) tended to increase the duration of the action potential and had a slow irreversible blocking effect. The resting potential was little affected. Potassium ferricyanide (60 m*M*) raised the threshold, blocked the action potential, and had its effects reversed with standard medium.

Of the reducing agents, both cysteine and 2-mercaptoethanol caused a decrease in the duration of the spike without affecting its height or the resting potential at concentrations of 100–150 m*M*. Above these concentrations there was deterioration in the action potential, but standard medium uniformly reversed the changes. Hydrazine caused deterioration at 90 m*M*, but this was reversible.

Mercaptide-forming reagents (*i.e.*, parachloromercuribenzoate, phenylmercuric acetate, mercuric chloride, fluorescein mercuric acetate, and sodium mersalyl (.1–1 m*M*)) were found to block the action potential and to cause (possible exception of phenylmercuric acetate) an increase in the resting potential (to −30 to −40 mV) in 8–45 minutes depending on the compound. These effects were reversible (90% recovery) with 2-mercaptoethanol, but not standard medium. Thiol alkylating reagents, iodoacetate, and iodoacetamide, concentrations to 10 m*M*, had no effect. Nor did N-ethylmaleimide. Metabolic inhibitors (cyanide, arsenate, arsenite) show no effect. Tests for ferredoxin-like structures in the membrane have been negative. Present efforts are directed at elucidating the suggested involvement of −SH groups in the axonal excitability mechanism.

A condition of temporary hyperthermia in a marine littoral snail. W. RUSSELL HUNTER AND MARTYN L. APLEY.

The littoral moon-snail, *Polinices duplicatus,* can be temporarily hyperthermic as a result of peculiarities of its environment and water physiology. Work (to be reported elsewhere) using inulin-labelled sea water has shown that 50–71% of the water within the pedal water-spaces can remain unexchanged for 72 hours. The retarded cooling of such snails and their contained water (up to 60–70% of live mass) was investigated, using thermistor probes in field and laboratory. At low tide in Barnstable Harbor, on a day when heating of the exposed flats was reduced by patchy clouds and a strong offshore wind, temperature in the upper 1 cm. of sand and in isolated pools ranged from 25.3° to 26.6° C., while incoming tidal water was 23.2° C. Snails crawling on these flats had internal temperatures ranging from 2.0° C. above to 0.3° C. below the sand temperatures. Those buried deeper than 1 cm. were cooler. With greater solar heating, the difference between the flats and incoming water could be as much as 6.5° C. Snails remained hyperthermic for an appreciable time after tidal submergence. Some were 0.7° to 1.0° C. above the surrounding water five minutes after being covered, and several were 0.2° C. above after 27 minutes. These field measurements involved relatively small specimens of *P. duplicatus* (25.3–28.6 mm. shells and 9–17 g. expanded weights). In laboratory experiments, larger expanded snails (46.5–49.9 mm. shells, 102–159 g.)

were transferred to sea water at temperatures 6.3–6.8° C. lower. These remained hyperthermic to the extent of 1.8° C. after 5 minutes, 0.9° C. after 10 minutes, and could thus remain 0.1° C. hyperthermic for 45–60 minutes. Empirically, heat is retained about as well as in an equivalent volume of confined but circulating water, but markedly less than in equidimensional foam latex rubber. This suggests some exchange of internal water during these experiments.

Such a temporary state of hyperthermia has little metabolic importance but is probably of considerable behavioral significance, since littoral moon-snails become active immediately after their inundation by the rising tide.

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Aspects of water physiology in the salt-marsh pulmonate snail, Melampus bidentatus. W. Russell Hunter and Robert T. Meadows.

It has been claimed that land snails and slugs (pulmonates) show rhythmic fluctuations in water content. Extensive recent work (to be reported elsewhere) on the land snails, *Helix pomatia* and *Otala lactea,* confirms the existence (even at 100% RH) of considerable fluctuations of period 5–15 days, but suggests that *regular* rhythms do *not* normally occur.

The local salt-marsh snail, *Melampus bidentatus,* belongs to the family Ellobiidae, being thus almost certainly related to the ancestral stock which gave rise to both land snails and fresh-water pulmonates. Numbered individuals of *Melampus* were maintained in controlled conditions in the laboratory, and weighed at intervals on a micro-torsion balance. Water content (as weight) varies with relative humidity (RH). Fifty snails, kept at humidities ranging from 80–100% RH and weighed at 12-hour intervals for 18 days, showed only random weight fluctuations (in extreme cases amounting to 11.4% of their maximum weight). Snails placed in 100% RH normally reach their weight maxima (*i.e.,* are wet-conditioned) in 1–2 hours. Wet-conditioned snails placed in 0% RH lose about 22.5% of their weight in 12 hours (then termed dry-conditioned), and survive. Few snails can survive >36 hours desiccation. Mean dry tissue weight (after death) is 45.4% of wet-conditioned live weight, 33.1% being inorganic shell. Two sets, each of 20 numbered *Melampus,* were regularly changed from 100% to 0% to 100% RH, and weighed, at 12-hour intervals (one set Day-dry, Night-wet; the other set Day-wet, Night-dry), for 11 days (22 changes). There were no water-content differences between the two sets, every individual fluctuating regularly to a dry-conditioned weight at about 77% of its wet-conditioned one. After this rhythm had been imposed, the snails were all transferred to 100% RH, where they gained their wet-conditioned (maximum) weight within two hours. Weighed at 12-hour intervals for the next seven days, they showed only small fluctuations (< 4%), and no trace of the imposed rhythm.

It appears that in *Melampus,* water content varies greatly, but is determined by environmental conditions, and shows no endogenous rhythmic fluctuations.

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Counteraction of Colcemid and heavy water on the organization of the mitotic spindle. Shinya Inoué, Hidemi Sato and Michael Ascher.

(1) Treatment of metaphase-arrested spindles of *Pectinaria* oocytes with Colcemid (N-desacetyl-N-methylcolchicine, a colchicine derivative with greater effectiveness and less toxicity), reduces spindle length and birefringence reversibly, confirming the action of colchicine reported on *Chaetopterus.* (2) D_2O increases spindle length and birefringence, as shown earlier in *Lytechinus* eggs. The effect is maximal at 45% concentration, is rapidly reversible, and can be repeated many times on the same egg. (3) D_2O delays Colcemid-induced reductions of spindle length and birefringence without altering actual rates of reduction. (4) The spindle length and birefringence begin to recover immediately when cells treated with Colcemid are washed with 45% D_2O or puromycin (10^{-5} *M*), provided spindle disappearance has not proceeded too far. (5) Cells which have completely lost their spindles by Colcemid treatment begin to recover their spindles after a lag period of one-half to one hour if washed with sea water or D_2O sea water. (6) The lag period could not represent the need for protein

synthesis since it is not affected by chloramphenicol (10^{-5} M) and is even reduced to one-third or one-quarter when cells are washed with puromycin (10^{-5} M) or actinomycin D (10^{-5} M). (7) These observations further substantiate Inoué's dynamic equilibrium model of the spindle.

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Denaturation of ovoverdin. CAROL JACOBS AND WILLIAM P. JENCKS.

Ovoverdin, extracted from lobster ovaries, was partially purified by precipitation by ammonium sulfate and by dialysis against distilled water. Denaturation by concentrated salts at pH 7.0 occurs in at least three stages: (1) The absorbance at 665 mμ drops approximately 15% (1.2 M $NaClO_4 < 5$ min.). (2) There is a larger decrease in absorbance over 15 to 60 minutes in more concentrated salt; in 2.4 M $NaClO_4$ the absorbance at 665 mμ decreases to 30% of the original value in a first-order process with a half-time of 15 minutes, while the absorption maximum at 465 mμ shifts to higher wave-lengths. (3) In concentrated denaturants (4.8 M $NaClO_4$) the 665 mμ peak disappears and the absorption of the prosthetic group at approximately 480 mμ is observed. The sensitivity to denaturation increases markedly with storage at 8° C. The concentrations of KI required to cause a 50% decrease in absorbance at 665 mμ at 30° C. were found to be 1.35 M and 0.75 M after 4 and 10 days at 8° C., respectively. The following salts had no visible effect on the color of ovoverdin in one hour: 5 M $NaNO_3$, 1 M Na_2SO_4, 3 M KCl, 4 M CsCl, 5 M NaCl, 2.5 M $(NH_4)_2SO_4$, 2.7 M $(CH_3)_4NBr$, 1 M KH_2PO_4, 2.5 M KF, 1.5 M Na_3 citrate. The following concentrations caused a 50% decrease after 30 minutes in the absorption at 665 mμ at 30° C. of a one-day-old preparation: 1.2 M NaSCN, 2.3 M NaI, 2.6 M $NaClO_4$, 2.9 M KI and 6.8 M urea, $\ll 2$ M Na benzoate, > 2 M Na acetate. Denaturation by urea and by heat is partially inhibited by KCl or Na_2SO_4. The rate and extent of denaturation by urea are independent of pigment concentration over a four-fold range, which is evidence against a reversible dissociation into subunits during denaturation. The results are generally similar to those for other proteins and for the activity coefficient of the model peptide, acetyltetraglycine ethyl ester.

Acid phosphatase staining reactions in the cercariae of the digenetic trematodes: Cryptocotyle lingua, Renicola thaidus and Parorchis acanthus. AARON JANOFF AND ARTHUR C. FORD.

The cercariae of certain species of digenetic trematodes are equipped with penetration glands which facilitate the entry of these larvae into the tissues of their intermediate hosts. We tested the possibility that granules contained in these gland cells are lysosomal in nature, in view of the clearly established digestive function of lysosomes in higher forms. Two species possessing penetration glands were stained for the lysosomal enzyme, acid phosphatase. These were: *Cryptocotyle lingua*, the cercariae of which were obtained from *Littorina littorea;* and *Renicola thaidus* whose cercarial stage was obtained from *Thais lapillus.* Larvae were fixed for 18 hours in ice-cold sea water containing 4% formaldehyde. Staining was carried out in Gomori's medium at pH 5.0 at 37° C. for one hour in the case of *C. lingua* and for three hours in the case of *R. thaidus.* Control specimens were stained either in the presence of 0.01 M fluoride to inhibit acid phosphatase or in the absence of substrate. In both species acid phosphatase reactions were not observed in penetration glands, but occurred instead in cystogenous glands, cells responsible for the secretion of the cyst wall surrounding the cercaria after its penetration into host tissues. A third species, *Parorchis acanthus,* which does not possess penetration glands but which does abound in cystogenous gland cells and in cells which secrete an adhesive agent for attachment of the cercaria to its substrate, also showed acid phosphatase reaction product in the latter cell type. Controls were negative in all three species. It was concluded that acid-β-glycerophosphatase is present in cystogenous glands of the cercariae studied. It may be that such cells are holocrine in function and that gland cell lysis facilitates the rapid release of secretory product during the encystment process.

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Specificity and quantitative aspects of the "renin-angiotensin" system in lower vertebrates. GABOR KALEY AND PETER C. DONSHIK.

The significance of the renin-angiotensin system as an important regulator of blood pressure and of aldosterone secretion in a number of mammalian species is well substantiated. Previous reports from this laboratory attested to the presence of renin in kidneys of a variety of lower vertebrates including reptiles, Amphibia and fishes. Inasmuch as fresh-water forms are faced with the task of sodium conservation, as opposed to marine forms, it was originally suspected that the renin-angiotensin system first appeared in fresh-water fishes. The finding of large amounts of renin in kidneys of marine organisms prompted us to examine for the presence of renin in a brackish-water reptile (turtle, *Malaclemys*), a marine teleost (*Tautoga onitis*) and a fresh-water teleost (catfish, *Ameiurus*). Whereas the intravenous injection of partially purified kidney extracts of the above species did not elevate the blood pressure of assay rats, incubation of kidney extracts with homologous plasma resulted in each instance in the formation of a vasopressor substance, the pharmacologic properties of which were indistinguishable from those of mammalian angiotensin. Criteria for angiotensin production included (1) inability to produce pressor substances by pH-inactivated kidney extracts, (2) abolition of pressor activity by incubation with chymotrypsin, and (3) measurement of plasma angiotensinase activity. The concentration of renin within kidneys of marine and brackish-water animals was considerably higher than that found in fresh-water forms and was comparable to that of rat kidneys. Whereas incubation of kidney extracts and plasma of marine teleosts resulted in angiotensin formation, no angiotensin was produced when they were incubated with either the plasma or kidney extracts of mammals, reptiles or fresh-water teleosts. Granulated juxtaglomerular cells, the presumed site of renin synthesis, were observed in most species studied.

Work completed in the Comparative Physiology Training Program under the support of NIH GM 1030.

Further purification of erythrophore-concentrating hormone from crustacean eyestalks. L. H. KLEINHOLZ AND FRANCES KIMBALL.

The crustacean eyestalk hormone (ECH) which concentrates the dispersed pigment in erythrophores of destalked test *Palaemonetes* has been obtained in one (Peak 4) of seven peaks after chromatography on columns of G-25 Sephadex. Peak 4 is heterogeneous in composition, a number of ninhydrin-staining spots being revealed by paper chromatography. When Peak 4 fractions are further purified by chromatography on G-10 Sephadex, six to eight peaks are resolved. ECH is found in the terminal fractions of the chromatogram, indicating that ECH is a small molecule. No loss in activity compared with that of non-trypsinized control extract occurs after incubation for 5 to 24 hours of samples of such ECH preparations with 0.1 mg. crystalline trypsin; incubation for 5 hours with 0.1 mg. crystalline chymotrypsin results in practically complete loss of activity. These results confirm the reported peptide nature of this hormone and indicate the probable presence of an aromatic amino acid residue in the molecule. Aliquots of such purified ECH, equivalent to 25 eyestalks of the original preparation, were chromatographed on paper; examination by short-wave ultraviolet light shows a deep-blue fluorescent spot and an absorbent spot (R_f being 0.24 and 0.50, respectively, in propanol-ammonia); eluates of these spots show no ECH activity on destalked *Palaemonetes*. No clearly positive staining is obtained with ninhydrin. A second UV-absorbent spot, closely behind the solvent front (R_f 0.86 to 0.92), is included in a Reindel-Hoppe staining area which shows tailing. Eluates of this region of the chromatogram show marked ECH activity.

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Some characteristics of liver cell membranes. ALBERT I. LANSING AND W. K. ZOLLINGER, JR.

Rat liver cell membranes were isolated by variations of the original technique of Neville and of the Emmelot and Benedetti procedure. Minced liver was homogenized in the cold in 0.001 *M* $NaHCO_3$, filtered, centrifuged at low speeds, and finally concentrated by use of a discontinuous sucrose gradient 20,000 *g*.

Membranes so prepared are separated from mitochondria which otherwise are a major source of contamination. These preparations, on examination with the electron microscope, reveal a frequent occurrence of desmosomes associated with trilaminal membranes.

Both DNA and RNA are present in the membranes, but the DNA is entirely removed by suspension of the material in Ecteola. It would appear that soluble DNA is adsorbed on the membranes and is selectively removed by Ecteola. RNA is consistently present in a concentration of about 6% (RNA: protein). These data are similar to those of Maulé, and analyses are being conducted to characterize the base composition of this RNA.

A study of γ-crystallin in the dogfish lens. S. LERMAN, S. ZIGMAN, J. TUTTLE AND W. FORBES.

A similarity between the distribution of soluble proteins in the dogfish and rat lens was previously noted. Changes in this distribution with aging have now been found by separating the α-, β-, and γ-crystallins by means of DEAE-cellulose column chromatography. The protein fraction which predominates in the lenses of young rats and dogfish is γ-crystallin which comprises about 65% (as determined by phenol reaction) of the total soluble proteins. The level of γ-crystallin decreases to approximately 40% of the total in older rats and dogfish, concomitant with a relative increase in the other soluble lens proteins. γ-Crystallin also becomes less soluble with aging.

Some of the chemical and physical properties of dogfish γ-crystallin have been elucidated. It is a basic protein containing high arginine, aspartic and glutamic acids, and an abundance of amino acids with hydrophobic side chains (determined by Moore and Stein amino acid analyses). The —SH content is 92 to 94 μmoles per gram of protein, a level which is much higher than that of the other soluble proteins.

The molecular weight of dogfish γ-crystallin is about 20,000, as determined by ultracentrifugation. The large number of hydrophobic groups may explain γ-crystallin's insolubility below 10° C.

γ-Crystallin appears to be composed of several different species, as it is in the rat lens.

By means of ultraviolet absorption analysis, it has been found that some of the tyrosines of dogfish γ-crystallin are not available to ionize when the protein is in alkaline solutions. This finding would indicate the presence of a tightly-packed core in the molecule in which phenolic groups of tyrosine are in close association.

The importance of this protein to maintenance of a transparent lens is presently under investigation.

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Cellular localization of clottable protein in Limulus amebocytes. JACK LEVIN AND FREDERIK B. BANG.

Limulus blood plasma, obtained free of amebocytes, is incoagulable. Following the aggregation and disruption of amebocytes in such cell-free plasma, the remaining liquid phase becomes coagulable. This material, defined as Pre-Gel, can be gelled by endotoxin. EDTA prevents gelation.

Amebocytes were removed by centrifugation from whole blood which was drawn directly into a solution of N-ethyl maleimide (NEM) at a final concentration of 5×10^{-3} *M* at 40° C. They were resuspended, repeatedly washed, and then placed in buffered sea water. Intact cells, thus obtained, were presumed to be free of plasma constituents. These cells differed from untreated cells by their lack of reaction to glass and their failure to send out filamentous processes. However, they slowly lost granules, and changed shape. Amebocytes, prepared in this manner, were disrupted by repeated freezing and thawing in buffered sea water. The resultant supernatant contained a substance, with a maximum U. V. absorption at 270 lambda, that was gelled by the endotoxin of a *Vibrio* species or *E. coli*. It is presumed to be a protein. It was stable when frozen but was inactivated by 30 minutes at 56° C. Approximately 14×10^{9} amebocytes yielded 1 mg. of material. Following gelation, the remaining supernatant was incoagulable.

This coagulable protein, therefore, is located within amebocytes, and is perhaps contained in the granules which pack the cytoplasm of these cells. It is likely that gelation caused by endotoxin does not require extracellular factors. The kinetics of this reaction resemble those observed when endotoxin causes the gelation of Pre-Gel, and it is similarly blocked by EDTA.

A preliminary survey of the properties of DNA during embryonic development. David Lipsky and Herbert S. Rosenkranz.

Studies currently underway in this laboratory revealed that the DNA's isolated at various stages of embryonic development of the American sea urchin, *Arbacia punctulata,* exhibited a gradual shift in the values of their buoyant densities as a function of differentiation. In view of the unusual nature of this finding and its relevance to our understanding of the function of DNA during embryonic development, this study was extended to include other biological systems. Results obtained thus far are indicative of the possibility that changes in the buoyant densities of DNA during embryogenesis are widespread phenomena. Thus it was found that the DNA's isolated from four-, seven-, ten-, and twelve-day old chick embryos had buoyant densities of 1.702_0, 1.701_6, 1.701_1 and 1.700_1, respectively. Similarly the DNA isolated from the sperm, fertilized egg and gills of the American oyster, *Crassostrea virginica,* exhibited buoyant densities of 1.699, 1.697 and 1.698 g./cm.^{3}, respectively. In addition, to the main band (1.699 g./cm.^{3}), the DNA from sperm also showed a satellite band of density 1.720 g./cm.^{3}.

The deoxyribonucleic acids from a number of other species were also isolated and these await analysis. However, the results reported here appear to support the previous findings with *Arbacia punctulata* and they may indicate that the DNA present in the developing embryo undergoes structural or compositional modifications.

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A search for new inhibitors of fibrin crosslinking. L. Lorand, T. R. E. Pilkington and Joyce Bruner-Lorand.

In 1962, Lorand, Konishi and Jacobsen demonstrated that the last step of vertebrate blood clotting is the crosslinking of fibrin by an enzymatic transamidation mechanism; amino groups of one fibrin molecule react with carbonyl functions of another to yield peptide-type of intermolecular bonds. Predictably, certain amines (glycine esters, hydroxylamine and hydrazine) inhibited the crosslinking process by being incorporated into fibrin and thus terminating the chain of polymerization. Such inhibitors seem to be of interest to protein chemistry and possibly also to pharmacology. They enable the exploration of the polymerizing centers of fibrin by specific labels. Further, experimental human thrombi formed in the presence of such inhibitors (though histologically indistinguishable from controls) lyse many times faster when exposed to urokinase.

Lobster plasma is so sensitive to these crosslinking inhibitors that, unlike vertebrate blood, it does not even gel in their presence; delay of lobster clotting time may be used to discern fine chemical requisites for such inhibition. New compounds examined (in order of effectiveness within each class) were: (a) *hydroxylamine derivatives:* O-(p-carboxy) benzyl hydroxylamine > O-benzyl hydroxylamine > γ-aminoxybutyric acid ethyl ester > β-aminoxypropionic acid > aminoxyacetic acid ethyl ester; (b) *lysine derivatives:* monotosylcadaverine > N$^{\alpha}$-tosyl-L-lysine methyl ester > ϵ-aminocaproic acid methyl ester > ϵ-aminocaproic acid; (c) *histamine derivatives:* histamine > 3-β-aminoethyl pyrazole > 3-β-aminoethyl 1,2,4-triazole > α-(β-aminoethyl) pyridine. The first compound listed inhibited lobster clotting in concentrations less than 0.1 m*M*.

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Comparison of ribosomal and nucleolar proteins by disc electrophoresis. Arne Løvlie and W. S. Vincent.

Proteins were extracted from isolated ribosomes and nucleoli of the starfish oocyte with 67% acetic acid or with 2 *M* LiCl in 8 *M* urea. The solutions were dialyzed against 8 *M* urea in 0.05 *M* acetate buffer, pH 5.3, and the soluble proteins separated by disc electrophoresis on gels made up at pH 4.8 and 8.9.

On the acidic gels the nucleolar extract gave 12 to 20 bands and the ribosomes 16 to 20. On the basic gels the nucleolar protein extracts gave a single band; the ribosomal proteins gave none.

Within the limits of the techniques used, only two of the bands from the nucleolar and ribosomal extracts could be shown to correspond in position on the gels. Therefore the results do not support the idea that the nucleolus of the starfish contains the complete spectrum of ribosomal proteins, nor intact ribosomes. Even if it could be shown that the bands which do appear to be similar were identical proteins, the relative amounts of these two proteins would be different in the nucleolus from that found in the ribosomes.

It is of interest to note that even though the proteins from these two sources do not appear to be the same on acrylamide gel electrophoresis, the basic proteins from the nucleolus do give approximately the same number of bands as the ribosomal proteins and show approximately the same mobility. This suggests that the basic proteins from the two sources possess similar size and charge.

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Melatonin reversibly augments mitotic spindle birefringence and inhibits the colchicine effect. STEPHEN E. MALAWISTA, HIDEMI SATO AND SHINYA INOUÉ.

In dermal melanocytes of the frog, *Rana pipiens,* a number of agents reversibly control dispersion and aggregation of melanin granules. One such substance is melatonin (N-acetyl-5-methoxytryptamine), a hormone which produces aggregation of dispersed granules, and inhibits dispersion of granules by other agents. Dispersion is thought to be accompanied by a reversible gel-to-sol cytoplasmic transformation. Melatonin, then, appears reversibly to support the gelated condition.

Colchicine produces dispersion of melanin granules in melanocytes, and interferes with operations that aggregate granules. The result is a dosage-dependent inhibition of aggregation, which may be explained by an effect on the equilibrium between protoplasmic sol and gel, where the most gelated conditions can no longer be developed. Melatonin inhibits this colchicine effect.

The mitotic spindle is a labile, gelated, birefringent structure, in which a dynamic equilibrium is postulated between oriented and disoriented material. The content of oriented material is measured in polarized light as retardation induced by spindle birefringence. Colchicine produces dissolution and contraction of the spindle.

Perfusion with melatonin (5×10^{-5} *M* to 1×10^{-4} *M*) of freshly spawned oocytes of the marine annelid, *Pectinaria gouldi,* resulted in increases of spindle birefringence by as much as 100%. The effect was readily reversible on removal of melatonin from the perfusate. Perfusion with the colchicine analogue, N-desacetyl-N-methylcolchicine (Colcemid, 1×10^{-5} *M*), resulted in a decrease in retardation and, within 6 to 12 minutes at room temperature, disappearance of the spindle. When melatonin (5×10^{-5} *M*) was perfused at the same time as Colcemid (1×10^{-5} *M*), the rate of decrease in retardation was diminished, and the ultimate disappearance of the spindle was delayed. Thus, melatonin, as well as colchicine, may influence similar biophysical systems in two widely dissimilar cell types.

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Determination of G-C content of DNA from Griffithsia globulifera. PETER C. MALONEY, A. E. BROOKS, VICKI L. GIORDANO, REGINA L. KORNBLITH AND MAIMON NASATIR.

Nuclei were isolated from the red alga, *Griffithsia globulifera.* DNA was extracted from the nuclear pellets by the following procedure: several washings with acetone-ethyl ether (3:1), and petroleum ether; extraction in 5% Duponol, 0.05 *M* sodium citrate, 0.007 *M* mercaptoethanol, pH 8.2; after centrifugation, cold ethanol was added to the supernatant; the precipitate washed with ethanol, and then dissolved in saline-citrate (0.15 *M* sodium chloride, 0.01 *M* sodium citrate, pH 7.0); the sample was deproteinized with chloroform-isoamyl alcohol (36:10): treated with RNase (5 mg./ml., at 37° C. for 30 minutes); and again deproteinized. Cold ethanol was added; the precipitate was washed with ethanol, and dissolved in a small volume of saline-citrate.

Ultraviolet absorbancy spectra gave values of *ca.* 0.77 and *ca.* 0.55 for the ratios of $OD_{240}:OD_{260}$ and $OD_{280}:OD_{260}$, respectively. Although diphenylamine-positive material could account for all the ultraviolet absorbancy, the DNA preparations still contained an orcinol-positive component. The DNA could not be separated from the orcinol-positive material by differential precipitation with cetyltrimethylammonium bromide and ammonium chloride under conditions which would separate DNA from alginic acid.

Melting curves were determined for DNA extracted from both diploid and male plants. The melting points were 85.5° C. and 86.4° C., respectively. These values correspond to 39.5% G-C in the DNA of the diploid plant, and 41.7% G-C in the DNA of the male plant.

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Isolation and composition of a water-soluble lipoprotein from Arbacia eggs. JULIAN B. MARSH.

Eggs of *Arbacia punctulata* were lysed in 0.05 *M* sodium phosphate buffer, pH 6.8, and centrifuged at 4° for 40 minutes at 100,000 × g. The clear supernatant was centrifuged overnight at density 1.21 (NaBr) and the top fractions combined and re-centrifuged. The final lipoprotein, comprising all the lipid in the supernatant, contained 15.9% protein, 8.7% carbohydrate, and 75.4% lipid. Its total echinochrome content was about 14% of the lipid, and 30% of it was dissociable on a column of G-200 Sephadex, from which the lipoprotein was excluded. No proteins of a molecular weight less than 500,000 were found, and this was confirmed by electrophoresis in a 5% polyacrylamide gel, which the lipoprotein did not enter. The protein moiety did not contain covalently-linked phosphate but the carbohydrate, which contained fucose, required hydrolysis for its release. A complete amino acid analysis of the protein was carried out, notable for the absence of methionine and the somewhat high (14%) content of serine plus threonine. The delipidized protein was soluble in 6 *M* urea. The lipid moiety contained 2.6% cholesterol (no esters) and 9.4% phospholipid, the remainder being echinochrome and triglyceride. An analysis of the fatty acids by gas chromatography revealed only even-numbered fatty acids up to C-22:1, of which 44% were saturated. In general, this lipoprotein resembles the low density lipoproteins of human plasma. It may represent a storage form of energy for the developing pluteus.

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Complex electrical responses from ganglion cells in the surf clam. DEFOREST MELLON, JR.

Electrical responses of a complex nature were recorded from surface cells in the anterior lobes of the visceral ganglion of the surf clam, *Spisula*. The response sequence from single cells, observed by intracellular recording, was similar following the arrival of electrically evoked volleys in a majority of the possible input pathways, including ipsilateral and contralateral excurrent and incurrent siphonal nerves, posterior pallial nerves, and cerebral-visceral connectives. In all cases the sequence consisted of a brief short-latency EPSP, one or two overshooting action potentials generated by the latter, and a subsequent IPSP lasting as long as one second. Recordings from semi-intact preparations showed that the observed sequence also occurs in response to transient mechanical stimulation of the peripheral sensory structures, and it therefore constitutes a physiologically valid régimen. The precise nature of the afferent pathways involved in the generation of the sequence was briefly examined. Recurrent axon collaterals from the impaled cells are not involved in the occurrence of the IPSP's, since the latter can appear even in the absence of impulses; moreover, cells never showed prolonged hyperpolarizations following impulses evoked directly by passing current through the recording electrode. If an identical afferent pathway were involved in the generation of both EPSP and IPSP recorded from a cell, then these responses should have identical thresholds to presynaptic stimuli. In fact, although both appear to increment to similar extents during each stepwise increase in voltage amplitude of an intensity series delivered presynaptically, identical thresholds have never yet been observed with respect either to electrical or mechanical stimulation. An alternative arrangement must therefore be sought—possibly one which depends upon separate afferent fiber populations with similar conduction velocities. Maintenance of the observed sequential relationships would therefore depend upon delay line mechanisms for the IPSP within the ganglion itself.

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Patterns of spontaneous activity in perfused lobster ganglion. MARTIN MENDELSON.

By perfusing with a buffered salt solution containing serum albumen and hemoglobin it is possible to support patterned, spontaneous activity in exposed, desheathed subesophageal ganglia of *Homarus americanus*. The activity monitored, of *ca.* 10 motor axons to the muscles of the scaphognathite, traverses two roots leaving the ganglion: one (posterior) contains motor axons innervating primarily elevator muscles; the other (anterior) primarily depressor muscles. The activity consists of rhythmic bursts of action potentials occurring alternately in the two roots, but with appreciable overlap. Virtually complete isolation of the subesophageal ganglion from the rest of the central nervous system does not produce noticeable long-term effects on the form or timing of the rhythmic discharge. Volleys entering the ganglion as the result of electrical stimulation of its roots, the circumesophageal connectives or the cord caudal to the ganglion cause a transient cessation or disorganization of the motor output. Recordings from the peripheral side of the posterior root gave no sensory responses to various stimuli. The anterior root readily subdivides into two bundles. One, smaller and more posterior, appears to contain all the motor fibers; the other none. Records from the peripheral side of the larger bundle occasionally reveal two or three units discharging in time with the observed contractions of the scaphognathite muscles. Units in the ganglion, penetrated with microelectrodes, are synaptically activated by stimuli to the scaphognathite roots. Some are excited, depolarizing PSPs lead to spikes. Units have been encountered which gave IPSPs upon root stimulation; one exhibited a burst of IPSPs at each cycle of the motor rhythm. The only maneuver which appears to produce consistent change in burst frequency is change in the rate of perfusion. Increase in the perfusion rate causes slowing or cessation of the spontaneous rhythm, decrease usually leads to acceleration.

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Site of action potential initiation in bipolar sensory cells of crabs. MARTIN MENDELSON.

Intracellular recording from somata of bipolar receptor cells of the PD joint organ of *Callinectes sapidus* reveals that action potentials in response to adequate stimuli rise abruptly from the resting potential baseline without discernible prepotential. Direct measurement of the threshold of the soma region, made by passing current across the soma membrane, indicates that at least 4–6 mV. depolarization is required. Yet mechanical stimuli of just subthreshold strength do not produce a generator potential of such magnitude. Hyperpolarization of the soma can block invasion into the soma of spikes elicited by either adequate or antidromic stimulation. The block may occur in either of two ways. Usually the response to an adequate stimulus is blocked first at a lesser level of hyperpolarization, leaving no electrotonically conducted sign; the antidromically evoked spike is not blocked until further hyperpolarization is applied and leaves a large electrotonically conducted potential. Less often, the spike evoked by adequate stimulation is blocked (still at a lesser hyperpolarization than the antidromic spike) leaving a small, all-or-none potential in its place. When, upon further hyperpolarization, the antidromic spike is blocked, the electrotonic potential is larger. It can be shown that the smaller blocked spikes, of the sort produced by adequate stimulation, are not accompanied by corresponding responses in the central axon. Only when the soma is permitted to produce a fully invading, overshooting action potential does a corresponding signal appear in the central axon. It is concluded that the distal processes of these bipolar cells are capable of supporting action potentials and furthermore that action potentials normally arise distal to the soma in response to mechanical stimuli.

Equipment used in this work was purchased under a grant, #NB 04588, from the National Institute of Neurological Diseases and Blindness, U.S.P.H.S.

Developmental and genetic studies on the compound ascidian, Botryllus schlosseri. Roger Milkman and Edward Therrien.

High-yield *in vitro* raising of embryos from fertilization to mature larvae is obtained by placing them on a disc of filter paper in a Syracuse dish. Larvae are transferred for settling on glass. After metamorphosis, oözooids have been grown in isolated culture using *Cyclotella nana* (centric diatom) and *Dunaliella* (phytomonad) for food. Colonies with dozens of zooids have so far been obtained, but no eggs. These colonies fare better than those brought in from the wild: since zooids grow in size as well as number during early colony development, a mass increase on the order of 1000-fold is indicated. Instant Ocean works as well as sea water for embryos, algae and *Botryllus* colonies.

Feulgen-staining of eggs, preceded by pronase removal of the cellular chorion and the blockade of cytoplasmic aldehydes with phenylhydrazine, indicates a haploid chromosome number of either 7 or 8. A variety of genetic crosses has been made. Great parental heterozygosity is indicated, making several generations of selfing desirable before extensive genetic analysis is undertaken. For this, egg production in culture is required.

Pairs of colonies which differ in color pattern have been allowed to fuse vascular systems. Maintenance of the original patterns has been confirmed. Subsequent removal of all zooids and buds, leaving only vascular system and matrix, resulted in extensive vascular budding. Two kinds of buds were seen in each experiment, resembling one parent or the other, with no intermediates or recombination. This absence of somatic hybrids shows that the anlagen of vascular buds are fixed rather than floating in the bloodstream. The formation, ultimately, of normal colonies from the simple vascular tissue attests to the great generative ability of some somatic cells in *Botryllus*.

Distributional aspects of the Cape Cod eel grass epibiota. J. Stewart Nagle.

The macrofauna, meiofauna, and epiflora of the southwest Cape Cod eel grass community have been studied for (1) horizontal and vertical distribution on individual plants, (2) organism interrelationships, and (3) areal differences related to physical requirements of the organisms.

It has been found that faunal abundance on individual plants varies with: (1) distance from the bottom, and (2) abundance of epiphytes. Animals which normally live on the surface of the sediment may also be found on eel grass, but their numbers generally decrease exponentially from the base of the stem. Fouling organisms, such as bryozoans and ascidians, live high up on the stem, but not where dense growth of epiphytes obstructs their feeding. Abundance of copepods and some genera of nematodes is related to epiphytic diatom concentration, since these animals apparently feed upon diatoms. Numerically, amphipods, snails, and the other nematodes show a relation to large epiphytes, mainly *Ceramium*, *Champia*, *Polysiphonia* and *Spyridia*. This relationship is somewhat mutualistic, as the epiphytes trap detritus which is consumed by the animals and used by some amphipods for tube construction. Moreover, the epiphytes conceal the amphipods from predators. The epiphytes benefit from the detritus feeding habits of the animals, for if the detritus is not removed the epiphytes will die. Variations in horizontal distribution relate to faunal occurrence on the large epiphytes. The tips of the epiphytes are occupied by snails and predominantly domicolous amphipods such as *Corophium*. These are apparently less vulnerable to fish predation than the more vagrant forms such as *Cymadusa* and *Microdeutopus*, which live near the eel grass stem among the lower portions of the epiphytes.

Macrofaunal forms show areal differences related to kinetic energy and salinity tolerances. The detritus-feeding amphipod *Gammarus* replaces its counterpart *Microdeutopus* in waters of estuarine salinity. In environments with high physical energy, the detritus-feeding amphipod *Corophium* replaces *Microdeutopus,* the diatom-feeding amphipod *Dexamine* takes the place of *Amphithoe,* and the snail *Lacuna* replaces *Bittium.*

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Seasonal aspects of Hadley Harbor benthic ecology. ROBERT H. PARKER, J. STEWART NAGLE, ANTHONY B. WILLIAMS AND ROBERT KAUFMAN.

Animal populations in Hadley Harbor benthic communities vary seasonally in numbers and dispersion. Populations of infaunal amphipods and mollusks increase markedly immediately after their breeding periods, decreasing to a minimum by the next breeding season. Coexistent and predominant infaunal animals have breeding cycles which are staggered, perhaps to avoid competition in larval settlement. However, infaunal animals exhibit constant annual dispersion, occurring in the same areas and at the same stations in summer and winter.

Mollusks epifaunal on eel grass show a summer maximum coincident with breeding periods, but also have high fall and winter maxima, unrelated to breeding. Eel-grass-dwelling amphipods not only exhibit the concentrated winter maxima of mollusks, but also have several submaxima throughout the year. These submaxima are related to the staggered breeding cycles of potentially competing species which breed throughout the year.

Eel grass epifauna are more concentrated at certain stations in the winter where the eel grass survives the winter kill. Since these organisms shift their population centers to areas of high winter eel grass cover, and many species virtually disappear from areas where the eel grass dies down, it appears that a migration takes place. Moreover, some eel grass forms, such as the amphipod, *Microdeutopus damnoniensis,* seem to be excluded from the shallow eel grass flats by high summer temperatures. With the reoccurrence of cooler fall temperatures these forms reappear in the shallow areas.

A relatively stable summer thermocline develops in the inner harbor, resulting in a reduction in oxygen below the thermocline, and ultimately the development of reducing conditions on the surface of the bottom. During this time the motile infauna, such as the pelecypods, *Yoldia limatula* and *Macoma tenta,* disappear from the area, migrating to more favorable oxygenated locations.

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Factors influencing sol-gel changes and activation of the egg of Arbacia punctulata. ARTHUR K. PARPART AND PHILIP J. MOORAD, JR.

Activation of the egg of *Arbacia punctulata* has long been known to involve cytoplasmic sol-gel transformations. The motion of echinochrome granules and cytoplasmic streaming have been used to determine the direction and state of these transformations. Various parthenogenetic agents will change the normal semi-sol to a semi-gel state without altering the cortical granules of the egg, *e.g.,* acids (above pH 3), hypo- to isosmotic and hyper- to isosmotic sea water, cysteine, low temperature (5° C.) to higher (20° C.), concentrated protein solutions in sea water (albumin, hemoglobin). Eggs exposed to these agents are activated and will undergo parthenogenetic cleavage with intact cortical layer and granules. Other agents induce a breakdown of the cortical granules under isosmotic conditions, which is followed by sol-gel alterations and full activation, *e.g.,* sperm, a number of non-penetrating non-electrolytes, papain, acids (pH 2 to 3). The factor common to both these types of agents is the change in the cytoplasm from a semi-sol to a semi-gel state. The gel that forms on activation is so stiff in the outermost 3 to 5 micra of the egg that all cytoplasmic streaming and echinochrome granule motion is stopped. Internal to this, the degree of cytoplasmic streaming is greatly reduced, though the semi-gel state is sufficiently loose to permit centration of the nucleus. Data obtained also suggest that the primary function of a spermatozoan in activation is the explosive rupture of the cortical granules which release a glyco-protein in a depolymerized state. This glyco-protein has a temporarily high osmotic pressure, equivalent to 20% albumin, and it withdraws water from the outermost region of cytoplasm leading to a local rapid gelation, thus initiating activation.

Instability of RNA formed during outgrowth of bacterial spores at low temperature. WILLIAM PERL AND HARLYN O. HALVORSON.

An unusual behavior of newly synthesized RNA during outgrowth of *B. cereus* strain T spores at 15° C. has been noted. First, actinomycin D, which stops DNA-dependent RNA synthesis, when administered (10 μg./ml.) at 90 minutes after initiation of germination (zero time) at 15° C. induces an almost complete breakdown ($>$ 80%) of RNA labeled with C^{14}-uracil from zero time. The main half-time of this decay is of order of 50 minutes. In contrast, the comparable experiment at 30° C. shows very little breakdown in the presence of actinomycin D. Experiments with pulse labeling followed by actinomycin D, at 30°, show the well-known phenomenon of the breakdown of the bulk of the newly synthesized RNA with a half-time of the order of 2 minutes. Second, when protein synthesis is inhibited during outgrowth at 15° C. by 100 μg./ml. of chloramphenicol, RNA labeled with C^{14}-uracil from zero time decreases after a few minutes. In contrast, at 30° C. only an accelerated increase in this RNA is noted. Puromycin produces qualitatively the same effects as chloramphenicol. The actinomycin D results are consistent with the hypothesis that at 15° C. most of the ribosomal RNA is unstable. For example, a delay in the completion of rigid, RNase-resistant ribosomal particles, such as by incomplete coating by ribosomal protein, could yield particles whose RNA is accessible to nuclease attack. The effects with chloramphenicol and puromycin can be understood if at 15° C. the system is dependent upon the synthesis of an enzyme which is temperature-sensitive and required for RNA synthesis.

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DNA and the embryonic development of the American sea urchin, Arbacia punctulata. HERBERT S. ROSENKRANZ.

We reported recently on the properties of the DNA's isolated from the egg, sperm and adult diploid tissues of *Arbacia punctulata.* It was found that the DNA isolated from the two haploid cells had a similar buoyant density of 1.700 g./cm.^{3}. This can be taken to mean that the DNA's from these sources have identical base compositions. On the other hand, the DNA derived from adult diploid cells exhibited a buoyant density of 1.702 g./cm.^{3}. If this difference in the density values of the DNA of haploid and diploid cells reflects a real shift in base composition, it is indeed a most unexpected finding. For this reason an investigation was undertaken to study the properties of the DNA isolated at various stages of embryonic development. It was thus found that a buoyant density of 1.700 g./cm.^{3} was exhibited by the DNA's isolated from the fertilized egg prior to cleavage, from the two- and sixteen-cell stages and from morulae. The DNA from blastulae had a density of 1.701 g./cm.^{3}, while the material isolated from gastrulae and plutei exhibited a density of 1.702 g./cm.^{3}. It was ascertained that these differences were real, by isolating specimens at each stage by two different procedures and analyzing each sample at least twice. These may indicate an active role for DNA in cellular differentiation.

This study was aided by a contract (Nonr 266(89), NR 103-574) between the Office of Naval Research, Department of the Navy, and Columbia University.

Preliminary characterization of a non-nucleotide macromolecule found in the DNA isolated from the sand dollar, Echinarachnius parma. HERBERT S. ROSENKRANZ.

Cesium chloride density gradient centrifugation of DNA isolated from *Echinarachnius parma* reveals the presence of two bands. The major band of density 1.700 g./cm.^{3} exhibits the properties commonly associated with DNA. The density of the second band (1.682 g./cm.^{3}) indicates that this material may be a co-polymer of deoxyadenylate-thymidylate. Further analysis, however, revealed that this material was resistant to DNase and to heat denaturation in the presence of formaldehyde, thus casting doubt on its nucleotide character. Several procedures were used in an attempt to isolate, in pure form, this unusual component. Fractionation of a DNase digest on Sephadex G-100 was the method finally adopted to achieve this purpose. The material so obtained did not exhibit a nucleic acid-like spectrum. It had a sedimentation coefficient of approximately 900S. Upon acid hydrolysis and electrophoretic separation, the presence of amino acids, reducing sugars and hexosamines was revealed. The exact nature and function of this material are under continued investigation.

This study was aided by a contract (Nonr 266(89), NR 103-574) between the Office of Naval Research, Department of the Navy, and Columbia University.

Mercenene: A preliminary investigation of the cytological effects of this anti-tumor agent extracted from Mercenaria mercenaria on the Krebs-2 carcinoma. M. ROSARII SCHMEER AND GRACE BEERY.

Mercenene, a growth-inhibitor extracted from edible molluscs, and in particular the quahog, *Mercenaria mercenaria*, has been studied *in vivo* and *in vitro* for the cytological effects it produces on the solid Krebs-2 carcinoma tumor (K-2) in CF1 mice and monolayer cultures of HeLa and normal human amnion lines grown on sterile glass. An extract was prepared as reported earlier, and injected subcutaneously into mice that had previously received 25–30 million ascites K-2 cells. Control or untreated animals received 0.25 ml. physiological saline in the same dosage schedule as the treated groups. On the eighth day after initial treatment was begun, tumors from all treated and untreated mice were excised and prepared for light microscopy investigation. Many tumors from repeated experiments were studied. Among the observations made, it was noted that the treated mice had smaller tumors, and that sections of these tumors showed a great infiltration of fibroblasts, fewer mitotic cells, less necrosis, small numbers of white blood cells as compared with control or untreated tumors, little polyploidy, normal nuclear-cytoplasmic ratio, and few cells with enlarged nuclei usually predominant in the K-2 tumor. HeLa cell cultures showed a 4+ degeneration in 48–72 hours after initial treatment with Mercenene, while normal human amnion cells showed little to no degeneration for the same period of time.

We wish to thank the Ohio State Health Laboratories, Columbus, Ohio, for HeLa cell lines and Dr. Bertha Bouroncle (University Hospital, Columbus, Ohio) for providing our laboratory with normal human cell lines. This research was supported by American Cancer Society Award T-361.

Mercenene: Growth-inhibitor extracted from Mercenaria campechiensis. In vivo and in vitro activity. M. ROSARII SCHMEER AND GRACE BEERY.

It has been previously reported that a growth-inhibitor called Mercenene was extracted from a species of edible clam, *Mercenaria mercenaria*. This clam is very abundant in Rhode Island and Cape Cod marine waters. Testing our theory that the growth-inhibitor may be due to some special feeding habit of the animal, we tested another species of *Mercenaria*, *Mercenaria campechiensis*, that is relatively abundant south of the Chesapeake Bay area. Water extracts of the fresh, raw clam were prepared in an identical procedure used for the preparation of extracts of *M. mercenaria*. Previously implanted CF1 mice received 0.25 ml. of various concentrations of the extract, each day for seven full days. Control, untreated animals had an identical dosage schedule, but received, in place of the extract, 0.25 ml. physiological saline. On the eighth day after initial treatment with Mercenene, the Krebs-2 carcinoma tumors were excised and weighed. Control animal tumors were also weighed at the same time. It was observed that a 25% concentration of the water extract induced the greatest regression and inhibition of tumor. *In vitro* investigations using the HeLa and normal human amnion cell lines suggest a toxic effect on the HeLa lines, with very little or no degeneration of the human amnion cell line.

The presence of Mercenene in an additional species of edible clam may indicate a selective feeding habit, or it may suggest that the clam ingests some agent or agents in the marine environment, changes it *in vivo,* and produces a chemically modified principle that possesses anti-cancer activity.

This research was supported by American Cancer Society Award T-361. We wish to thank Dr. Bertha Bouroncle (The Ohio State University Hospital, Columbus, Ohio) and the Ohio State Health Laboratories, Columbus, Ohio for human amnion and HeLa cell lines.

Deep-Sea Ectoprocta from 300–4680 m. on the Gay Head, Massachusetts–Bermuda, U. K., transect. Thomas J. M. Schopf.

Eight collections of ectoprocts taken at depths between 300 and 4680 m. on the Gay Head, Massachusetts–Bermuda, U. K., transect, through the courtesy of Dr. H. L. Sanders, Woods Hole Oceanographic Institution, have been examined. Two other collections of deep-sea ectoprocts, taken during the U.S.G.S.—W.H.O.I. Continental Shelf Program, kindly were made available by Dr. R. L. Wigley, Bureau of Commercial Fisheries, Woods Hole, Massachusetts. Of these ten collections, four are from the Continental Slope (150–1050 m.), three from the Upper Continental Rise (1050–4350 m.), one from the Lower Continental Rise (4350–5200 m.), one (4667 m.) from near the base of the Bermuda Pedestal Depositional Terrace, and one (2500 m.) from the Bermuda Pedestal.

These collections include eight (possibly 11) genera and 14 (possibly 17) indigenous species. None of the definitely indigenous species is present in the writer's extensive collections from the adjacent continental shelf. The continental slope fauna includes four species: *Ushakovia* sp. A (300 m., 400 m., 466.7 m., 500 m.), *Kinetoskias* sp. cf. *K. cyathus* (300 m.), *Salicornaria* sp. A (466.7 m.), *Cornyoporella* sp. A (466.7 m.). *Bifaxaria* sp. A (3820 m.) and *Bugula* sp. A (2000 m.) occur only on the Upper Continental Rise. Three other species occur on both the Upper and Lower Continental Rise: *Levinsonella magna* (3820 m., 4680 m.), *Salicornaria magnifica* (3820 m., 4680 m.), *Salicornaria* sp. B (2864 m., 4680 m.). Four species occur only on the Lower Continental Rise: *Cellularia* sp. A (4680 m.), *Kinetoskias cyathus* (4680 m.), *Kinetoskias* sp. A (4680 m.), *Kinetoskias* sp. B. (4680 m.).

The ectoproct collection from the base of the Bermuda Pedestal Depositional Terrace (4667 m.) contained only one fragment of *Salicornaria* sp. C, and six fragments of two cyclostome species, *Crisia* sp. A (ovicell present) and an undetermined cyclostome species. Although it is possible that the two cyclostome species are indigenous (and thus the first representatives of that order to be found living in water deeper than 3000 m.), it seems likely that they were introduced by turbidity currents as have been some sediments in this area.

The Bermuda Pedestal station (2500 m.) yielded one colony of a dead *Alcyonidium* sp. *cf. A. mamillatum,* with the zooecia in part filled with sediment and encrusting a dead hydroid. In view of the geologic setting, it seems likely that this specimen was removed to this great depth by turbidity currents. If later collecting shows this occurrence to be indigenous, then it is apparently the first ctenostome recorded from a depth greater than 1000 m.

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Ectoproct (Bryozoa) distribution on the Atlantic Continental Shelf from the Hudson Canyon to Nova Scotia. Thomas J. M. Schopf.

The eastern Continental Shelf of North America from the Florida Keys to Nova Scotia has been sampled on an 18-km.^{2} grid to serve geological and biological purposes by the U.S.G.S.-W.H.O.I. Atlantic Continental Shelf Program. In the northern third of the shelf, approximately from the Hudson Canyon to Nova Scotia, about 650 stations have been sampled. Of these, about 250, or nearly 40%, yielded identifiable ectoprocts. Of these 250 collections, 120 have been examined in detail and yielded a total of 63 ectoproct species. These include 50 cheilostomes (28 ascophorans, 22 anascans), 10 cyclostomes, and three ctenostomes. The vast majority of these species have been previously reported from waters off eastern North America, but a few (*e.g., Smittina smitti* (Kirchenpauer)) are new to the fauna. The average number of specimens per station yielding ectoprocts is estimated as fewer than 30 and the average number of ectoproct species per station is 5.5 (range of one to 35).

Stations yielding ectoprocts are mainly grouped in four areas. These are on the Nova Scotia Shelf, the northeast quarter of Georges Bank, the Nantucket Shoal area, and along the western border to the Jordan Basin, south of Mt. Desert Island. In general, ectoprocts are absent from basins, *i.e.*, areas of prevailing clay sediments and absence of hard substrates. A strong positive correlation exists between areas of coarse substrate (more than 25% gravel or more than 75% sand) and ectoproct occurrence.

The ectoprocts of the Nova Scotia Shelf include the species most typical of Arctic areas of those studied. Many of these colder water forms also are found in the Gulf of Maine. The colder Nova Scotia waters are recycled and warmed in the Gulf of Maine because Cape Cod and Georges Bank form a land barrier. Thus, the ectoproct fauna of Georges Bank seems more like that of the warmer water of the Nantuckt Shoal region than of the closely adjacent Nova Scotia Shelf.

In addition to substrate and bottom temperature, the roles of bottom salinity, oxygen, and rate of sedimentation were examined. None of these factors varies systematically with ectoproct distribution and hence are less suitable for understanding the basis for species distribution.

Supported by a grant from the Ford Foundation to the MBL Systematics-Ecology Program.

Entoproct records from northeastern United States: Loxosomella varians Nielsen and L. minuta (Osburn). THOMAS J. M. SCHOPF AND JOSEPH L. SIMON.

Loxosomella varians Nielsen is recorded for the first time from North America. Previous records are from Norway and Iceland. It inhabited the inter-parapodial grooves and branchiae of the polychaete annelids, *Nephtys incisa* from off Nashawena Island, Buzzards Bay, Massachusetts (41°26′38″N, 70°52′35″W), July 7, 1964, and *N. ciliata* and *N. discors* collected at Crowe Neck, North Trescott, Maine (44°53′N, 67°8′W), July 31, 1965. In each case buds were present and hence the entoproct was reproducing.

Twenty-two specimens of *Loxosomella minuta* (Osburn) have been found of one individual of the sipunculid, *Phascolion strombi*, living in the shell of the small gastropod, *Nassarius trivitatus*, from Quissett Harbor, Massachusetts (41°32′N, 70°38′W) July 19, 1965. We are indebted to Dr. J. Gage, Woods Hole Oceanographic Institution, for calling our attention to this sipunculid. This is the first record of *L. minuta* in this, the type area, since Osburn's original description of 1912. Eight tentacles occurred on each of the ten specimens sufficiently relaxed to permit a count. The average length and width of the calyx of ten specimens were, respectively, 0.30 mm. (0.24–0.46 mm.) and 0.22 mm. (0.19–0.33 mm.). The average total length of six complete specimens was 0.47 mm. (0.44–0.59 mm.). This size range is close to Osburn's description of an average length "not more than a third of a millimeter, while the largest specimen seen measure[d] under one-half millimeter." One or two buds were present on 13 specimens. Hence the specimens examined are adults and it is concluded that *L. minuta* is smaller than most other entoprocts.

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The action of phenotropic drugs on melanophore activity in the sand flounder, Scopthalamus aquosus. GEORGE T. SCOTT AND GARY GARDNER.

Additional physiologically active pharmacological agents were assayed for local melanophore pigment-aggregating or dispersing activity by subcutaneous injection in the opercular area. Minimum effective doses were determined by noting a positive response in three or four out of five fish.

The most active melanophore pigment-aggregating agents were epinephrine, norepinephrine, isopropylnorepinephrine, dopamine, melatonin and serotonin with effective doses ranging from 0.02 to 0.10 microgram. Three energizers, phenelzine, pheniprazine and etryptamine, were active at 0.3, 0.4 and 2.0 micrograms, respectively. Iproniazid and isocarboxazid were inactive. The metabolic products of epinephrine, metanephrine and mandelic acid, were inactive. Dihydroxyphenylalanine (Dopa) was also inactive.

Pretreatment with pyrogallol (5 mg. per kg.), an inhibitor of catechol-o-methyl transferase, resulted in marked potentiation of certain catechol amines. Pretreatment with iproniazid, a monoamine oxidase inhibitor in a dose range of 10–200 mg. per kg., had no effect.

Of the large number of drugs causing localized melanophore dispersion, the phenothiazine tranquilizers were most active, in an effective dose range of 0.08 to 5 micrograms. The most active members of this group were fluphenazine, perphenazine, fluorophenazine and thiopropazate. Pretreatment with pyrogallol (5 mg. per kg.) raised the effective dose of these agents by one to two orders of magnitude but had no influence on the other phenothiazines examined (trifluoperazine, prochlorperazine, acetophenazine, chlorpromazine, mepazine, proketazine).

These data suggest the following: (1) A catechol amine, metabolized by catechol-o-methyl transferase, is the physiological transmitter. (2) The most active phenothiazines, whose effective dose is raised by pyrogallol, impose a blockade on the action of the normal transmitter which functions to maintain the fish in a blanched condition.

Studies involving chromatographic techniques are in progress to identify chemically a material extractable from fins of white-adapted fish which causes marked localized pigment aggregation when injected subcutaneously. Such material is presumably of neurosecretory origin.

The research was supported by Grant MH 03903 from the National Institute of Mental Health to Oberlin College.

Early development of Lepidonotus sublevis Verrill, a commensal polychaete. JOSEPH L. SIMON.

Lepidonotus sublevis adults were obtained by cracking the shells of their host animals, *Pagurus pollicaris.* Scaleworms inhabit both the columella and body whorls of the hermit crab shells. Eggs and sperm were obtained by puncturing the body wall at the bases of the parapodia of the female and male worms, respectively. The egg has a wrinkled membrane prior to fertilization and is 100 μ in diameter. Sperm are of the typical "primitive" annelid type. They possess a spherical head about 2.6 μ in diameter, a middle piece about 1 μ long, composed of 4 mitochondrial spheres, and a tail about 50 μ long. A tiny acrosome is present. Eggs were washed several times in filtered sea water, to remove contaminating coelomic fluid, and were allowed to stand 20–60 minutes prior to insemination. When eggs and sperm were mixed, 50% to 85% fertilization was obtained. Cleavage is spiral and equal. Development at temperatures of 21–23° C. occurred at a very rapid rate: two-cell stage, 45–60 minutes; swimming blastula, 4 hours; early trochophore, 11 hours; late trochophore, 24 to 72 hours. The trochophore is characterized as follows: 115–125 μ long; unpigmented except for one pair of black eyes; complex ciliation, including an apical tuft, akrotroch, prototroch, oral cilia, long lateral feeding cilia, and neurotroch. Trochophores were positively phototactic and actively fed on unicellular algae. Attempts to rear larvae beyond trochophore were unsuccessful.

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Abnormalities in developing sea urchins induced by certain radiomimetic chemical compounds compared with those induced by irradiation, with special reference to nuclear damage (wth motion picture). CARL CASKEY SPEIDEL AND RALPH HOLT CHENEY.

We have subjected gametes and zygotes of *Arbacia punctulata* to three radiomimetic compounds, colchicine, nicotine and caffeine, and to two types of irradiation, gamma (cesium 137 irradiator) and 2537 Å ultraviolet. Severe, mild and intermediate dosages of each agent were used for treatments of eggs alone and sperm alone, followed by fertilization with normal gametes, and also treatments of zygotes from first cleavage to young pluteus. Resemblances and differences in the resultant progeny were recorded by cinephotomicrography and analyzed with the major aim of determining the relative damage to nuclear and cytoplasmic constituents caused by the five different treatments.

Widely *different* dosages to sperm alone and to eggs alone were required to induce approximately *equivalent* damage to the respective progeny. Our rough estimate of such dosages was in the *proportion* of 1 for gamma to sperm to 1.5 for gamma to eggs; 1 to 4 (or more) for ultraviolet; 10 to 1 for nicotine; and 50 to 1 for colchicine. Caffeine to sperm induced little or no damage in marked contrast to caffeine to eggs or zygotes. Damage induced by treatment of sperm alone is probably ascribable to *nuclear* damage inflicted by radiomimetic compounds or radiation types; that following treatment of eggs alone is ascribable to *both nuclear and cytoplasmic* damage.

Colchicine, which profoundly affects distribution of chromosomes during cell division, is of special significance in the interpretation of observed abnormalities. Abnormalities in progeny arising from colchicine-treated *sperm* are *like* those induced by irradiated *sperm*. Both gamma and 2537 Å ultraviolet rays damage the sperm nucleus with its chromosomes, rich in gene-coding DNA. The implication is strong that colchicine similarly damages the sperm nucleus. We suggest also that colchicine probably damages the unfertilized egg nucleus, the two pronuclei, and the syngamic nucleus of first cleavage.

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Glycerination-induced A-band contraction in invertebrate striated muscle. R. E. STEPHENS.

The phenomenon of A-band contraction or shortening has been reported for a number of invertebrates: *Limulus polyphemus* (horseshoe crab), *Balanus aquila* (barnacle), *Pecten irradians* (scallop), *Homarus americanus* (lobster), and *Cambarus virilis* (crayfish). The effect has been used as an argument against the sliding filament contraction model. Previous studies by the author have indicated, however, that these muscles operate by a mechanism consistent only with the sliding filament model when subjected to ultraviolet microbeam analysis. Intrinsic birefringence studies indicate homogeneous distribution of material within the A-band, regardless of A-band length.

All previous studies have been carried out on glycerinated myofibrils. If one glycerinates *Limulus, Balanus, Pecten, Homarus* or *Cambarus* muscle for only one hour and then dissects myofibrils into an EDTA relaxing medium, one cannot observe A-band contraction. Glycerination for longer periods produces myofibrils which show progressively greater A-band shortening upon ATP contraction. Similarly, fixation of fresh tissue at various lengths reveals muscle of varying sarcomere length but constant A-band length, in accord with the sliding filament model.

If rabbit psoas muscle is treated with used glycerol from the invertebrate glycerinations, the rabbit myofibrils, upon contraction with ATP, show the A-band anomaly. Brief trypsin treatment of rabbit psoas myofibrils causes a marked decrease in A-band length upon ATP contraction.

It can be concluded, therefore, that the phenomenon of A-band contraction is dependent upon length of glycerination and, without glycerol treatment, the A-bands of these invertebrate muscles remain constant. All of the muscles show heavy bacterial contamination and, in light of the trypsin effect, the shortening phenomenon could conceivably be due to enzymatic digestion of the sarcomere components, permitting a homogeneous shortening of the A-bands upon contraction of the weakened structure.

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Photo-receptivity of the Golfingia cerebral ganglion. WILFORD P. STRATTEN.

A preparation of the ventral retractor muscles with the ganglion intact was isotonically suspended under a tension of one gram. The preparation was in a sea water bath and the effect of light incident upon the preparation was studied after an appropriate dark-adaptation. High intensity white light invariably caused contraction of the retractor muscles regardless of the area of the preparation illuminated. At low intensities, however, it was necessary for the cerebral ganglion to be stimulated to cause any response.

To get a consistent response to a stimulation of the ganglion it was necessary for the stimuli to be 10 minutes apart. There was no summation of response to consecutive stimuli. If the interval between stimuli was decreased, the second response decreased. There was no second response if the second illumination was within one minute of the first. For a full response it was necessary to follow any spontaneous contractions by at least three minutes.

The response to light stimulus was almost immediate contraction of the retractor muscles. A full contraction lasted on the order of 40 seconds, with two-thirds of that time being the relaxation time.

With an increase in the intensity of duration of the light stimulus there was an increase in the magnitude of contraction. A continued illumination gave a response similar to that of a five-second stimulus. There was no change in spontaneity. There was no "off" response after two hours of exposure.

The spectral sensitivity was studied in 25-millimicron steps of equal energy from 350 to 800 millimicrons. There was a definite peak of response at 500 millimicrons. The larger the deviation from 500 millimicrons the smaller the response.

This work was supported by the Comparative Physiology Training Program, U.S.P.H.S. GM 1030.

Studies on digenetic trematodes of the family Notocotylidae. HORACE W. STUNKARD.

Paramonostomum alveatum (Mehlis in Creplin, 1846) Lühe, 1909, one of the notocotylid trematodes, is a widely distributed parasite of ducks, geese, swans and related water-fowl. The life-history of the species was discovered by Mme. Kulachkova (1954), following mass mortality of chicks of the eider-duck, *Somateria* sp. Young birds, less than two weeks old, harbored up to 50,000 mature worms in the intestine, the wall of which was inflamed and the epithelium largely denuded. The asexual generations of the parasite were found in the snail, *Hydrobia ulvae,* and the cercariae encysted on the shells of the snails from which they had emerged.

During the past three summers, over 4000 specimens of *Hydrobia salsa* (Pilsbry, 1905) have been examined for infection by larval trematodes, and at least four species of notocotylid cercariae have been recognized. They are representatives of three distinct groups, distinguished by the form of the anterior portion of the excretory vesicle and designated the Monostomi, Imbricata and Yenchingenesis groups by Rothschild (1938). The infected snails were identified by isolation: a tedious task because the snails are small, the cercariae are large and the number liberated is small, two to ten in 24 hours. Often an infected snail will not shed for three or four days. For feeding experiments, it is necessary to isolate the snail and determine which of the species is harbored, since the cercariae encyst promptly, and after encystment, specific determination is not feasible. Cercariae of one species of the Monostomi group emerge between 10 AM and 2 PM. They are photopositive, swim from a few minutes to three or four hours, and then encyst. Cysts of this species have been fed to laboratory-reared ducklings and adult worms recovered eight days later. The worms are identified as *Paramonostomum alveatum.* Work on the other species is continued.

Investigation supported by NSF, GB-3606.

Further electron microscopic studies of the pigmentary system of the squid (Loligo pealeii Les.). GEORGE SZABÓ.

The pigmentary system of the squid comprises (1) the chromatophore system of the skin; (2) the pigmented epithelium of the eye; (3) the iridiocytes; (4) the ink gland and the ink sac.

(1) The chromatophore system consists of a pigment cell surrounded by muscle cells. It is innervated. The pigment cell contains a large, lobated nucleus, some granular endoplasmic reticulum, Golgi complex, mitochondria and vacuoles of varying sizes, including pinocytotic vesicles. The ommochrome pigment is located in large vacuoles. In glutaraldehyde preparations these vacuoles contain several small granules, whereas in osmium-fixed preparations these vacuoles are filled with a solid mass of dense material. No fibers have been found inside the chromatophores, but the cell is surrounded by a tightly woven network of collagen fibers embedded in a strongly osmiophilic material. The fibers of the muscle cells are inserted in between the folds of the membrane of the contracted chromatophore. There is a Golgi zone and some granular endoplasmic reticulum in the muscle cells. Large mitochondria are also

present. (2) The pigmented epithelium of the eye is similar to that of the vertebrates, although the pigment is ommochrome. (3) The light-reflecting iridiocytes are located under the eyes, under the chromatophores and around the ink sac. They contain a large number of electron-dense lamellae. These lamellae (iridiosomes) around the eye and the ink sac show no internal structure, but in the skin the lamellae consist of small granules in a helical arrangement. This row of granules alternates with membrane-bound supporting (?) structures of the same width. (4) The ink gland is a cavernous organ consisting of leaves of glandular epithelium. Each leaf has a central connective tissue stroma with blood spaces, smooth muscle cells and nerves. The columnar epithelium, bordering this stroma on both sides, consists of typical secretory cells. The pigment (melanin) is formed in vacuoles of varying sizes. The ink cells have microvilli and may have cilia. The ink cells are interdigitated and show typical septate desmosomes.

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Permeability change during procaine action potential in the crayfish abdominal flexor muscle fibers. KIMIHISA TAKEDA.

The normally graded responses of the muscle fibers were converted into conducted prolonged spikes by applying 10^{-3} g./ml. procaine. The spikes were essentially independent of the nature of the anion in the saline (Cl or propionate) and could be evoked in Na-free solutions providing Ca or Ba was present. The peak overshoot in normal crayfish saline was about 10 mV and increased 24 mV for a 10-fold increase in Ca, maintaining the solution isosmotic by removing Na. In Na-free solutions the comparable slope was 33 mV. The spikes exhibited a plateau with slow repolarization. When the membrane potential attained about −10 mV in the normal saline the repolarization was accelerated. The initiation of the falling phase occurred at more positive levels of the membrane potential on increasing Ca, and the spikes shortened correspondingly, both changes being linearly related to log Ca. The rising phase of the spike is associated with a brief large inward current (as measured with an extracellular pore electrode) which appeared to be due to Ca influx. Early in the plateau the conductance (measured by brief pulses) remained 2- to 3-fold higher than that of the resting membrane, presumably due to a remnant increase in Ca permeability. Late in the plateau a slow increase in permeability developed, reaching a maximum early in the falling phase, and declined. This appears to be due to a delayed increase in K permeability which results in repolarizing electrogenesis and termination of the spike. Mn in low concentrations suppresses the spike and the initial increase in conductance. The late conductance increase is almost unaffected, but arises earlier with increasing current-induced depolarization. Thus, the higher voltages of the spikes on increasing Ca probably also institute earlier K activation and this can account for the abbreviation of the spike.

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Absence of hyperglycemic response at or after ecdysis in Xiphosura polyphemus. HENRY TAMAR.

Groups of 6 small limuli (carapace width 6.2–8.2 cm.) were selected either for a hard exoskeleton or a soft one with resilient carapace. A 0.5-cc. pre-injection blood sample was drawn from the pericardial sinus of each specimen, and then 0.5 cc. of filtered sea water was injected at the same site. After three hours (at 26.5°–30° C.) a post-injection pericardial sinus blood sample of over 1.5 cc. was drawn. The animals had been starved for 6-17 days, but were crowded until one or two days before use. No specimens with any break in the exoskeleton were employed. The site of injection was pre-dried and above the level of the sea water in each bowl containing an animal. All bowls were covered. Blood glucose level was determined with the enzymatic Glucostat reagent and a Beckman DU spectrophotometer.

The average pre-injection blood glucose level for 30 limuli with a hard exoskeleton was 4.0 mg./100 ml., s.d. 2.1. The post-injection average for 24 of these hard-skeletoned animals was 20.1 mg. glucose/100 ml., s.d. 10.4. Their average increase in blood glucose was 15.9

mg./100 ml. The pre-injection average of 24 soft-skeletoned specimens was 2.6 mg. glucose/100 ml., s.d. 1.4, and their post-injection average was 1.0 mg./100 ml., s.d. 0.8. This represents an average drop of 1.6 mg. glucose/100 ml. of fluid. The differences between the means for post-injection hard and post-injection soft, pre-injection hard and post-injection hard, and pre-injection soft and post-injection soft animals were significant beyond the 0.001 level. The difference between the means for pre-injection hard and pre-injection soft specimens had significance exceeding the 0.01 level. Apparently the appearance of hyperglycemic response in *Xiphosura* can be dependent on the stage of the molting cycle.

Deep gratitude is expressed to Dr. L. H. Kleinholz for basic techniques and numerous valuable suggestions. Supported by PHS Grant 5T1 GM 1030-03.

Species-specific differences of Ampelisca (Amphipoda) as demonstrated by starch gel electrophoresis. SISTER M. GABRIEL TREBATOSKI, ERIC L. MILLS AND IRWIN W. SHERMAN.

Populations of organisms that appear to be a single species have in some cases been shown to consist of distinct and separable populations, each reproductively isolated so that they may be considered sibling species. Classical cases are described for *Drosophila* and more recently for the holothurian *Thyonella* (Manwell and Baker, 1963). This can now be extended to some amphipods in the genus *Ampelisca. A. holmesi, A. vadorum* and *A. abdita* are tube-dwelling, bottom forms that occupy different benthic ecological niches, and in the case of the latter two species are only slightly different in their morphology (Mills, 1964). Biochemical comparisons of the species were made, using the zymogram technique (starch gel electrophoresis followed by histochemical visualization of enzymes). Extracts of animals were prepared by homogenization followed by sonication and centrifugation. Starch gel electrophoresis was essentially by the method of Smithies (1955, 1959). Species-specific patterns for all three species were obtained for non-specific esterase, alkaline phosphatase and malic dehydrogenase. Enzymatic patterns obtained are consistent with the morphological and ecological separation of *A. vadorum* and *A. abdita* into distinct species.

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Apholate effects on early development of Arbacia. LAWRENCE R. VALCOVIC AND DANIEL S. GROSCH.

Apholate, an ethyleneimine-type alkylating agent, has been found to be an effective chemosterilant in programs of insect control. Other laboratories have investigated dipteran chromosomal aberrations and we have studied genetic effects on the parthenogenetic parasitic wasp, *Habrobracon.*

Arbacia punctulata was used in these experiments because its gametes have featured in classic experiments on delayed division and spindle structure. In concentrations of 1%, 2%, and 5%, apholate was injected into adult females. Eggs were gathered after a 24-hour incubation and fertilized with normal sperm. Average time delay for first cleavage was 23, 30, and 40 minutes, respectively, for the three concentrations used. Delay persisted throughout cleavage and ranged from one to 10 hours at 28 hours. Embryonic lethality increased after 12 hours, and reached 70% in the 5% treatment groups.

Preliminary experiments of 20-minute incubation with alanine-C^{14} at two and 9 hours after fertilization showed no decrease in uptake of label by treated embryos.

First cleavage delay in embryos derived from treated sperm was approximately twice that obtained from treated eggs, while delay in embryos from treated eggs and sperm was not significantly greater than from treated sperm alone.

These results extending observations to a marine echinoderm are in agreement with studies on insects. Delayed division with full recovery is explainable under the hypothesis that alkylation of nuclear DNA is reversible. Cytotoxicity resulting from higher doses is expected, since a high degree of alkylation will render DNA non-functional and any such material which is drawn into the mitotic process is likely to be fragmented. Damage to chromatids immediately before cell division can lead to anaphase bridging and cells containing these abberations are not likely to survive.

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Properties of the DNA isolated from sperm of Arbacia punctulata treated with hydroxyurea. JOHN M. VAN PRAAG, ERIC J. SIMON AND HERBERT S. ROSENKRANZ.

Previous studies with bacterial and mammalian cells showed that hydroxyurea (HU) is a specific and reversible inhibitor of DNA synthesis. In an effort to gain some understanding of the mechanism of action of this drug it was considered of interest to find out whether a direct *in vivo* effect of HU on the DNA contained in sperm of *Arbacia punctulata* could be demonstrated. For this purpose, sperm were exposed to a range of HU concentrations. After pre-determined intervals the drug was removed and a small portion of the sperm was used to determine their ability to fertilize normal eggs. The remaining sperm served as a source of DNA. The buoyant densities, as determined by CsCl density gradient centrifugation, were used to characterize the DNA specimens. It was thus found that whereas the DNA isolated from untreated sperm exhibited a unimodal gaussian distribution with a mean density of 1.700 g./cm.^{3}, the DNA isolated from sperm rendered infertile by treatment with 10^{-2} M HU for 7 hours was bimodal with a major non-gaussian band of density 1.702 g./cm.^{3} and a minor component of density 1.719 g./cm.^{3}. Very similar results were found with the DNA of newly-formed sperm obtained from animals that were exposed to 10^{-4} M hydroxyurea for 4 days.

These findings are consistent with the possibility that in *Arbacia* sperm the DNA is linked to a proteinaceous material which is removable by HU. With the loss of this protein the buoyant density of the DNA would be expected to increase. It may also be that the component of density 1.719 g./cm.^{3} is composed of single-stranded DNA segments which in the untreated sperm are linked to the double-stranded portion by non-nucleotide linkages and hence are not apparent upon CsCl density gradient centrifugation.

It is hoped that current studies dealing with the *in vivo* and *in vitro* effects of HU on the physico-chemical properties of the DNA of the sperm of *Arbacia punctulata* will provide additional data in support of this hypothesis.

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Transcellular effects induced in centrifuged Arbacia egg cells by locally applied ultrasound. FLOYD J. WIERCINSKI.

Ultrasound affords a means of probing the interior of living cells and tissues without breaking the cell membrane. *Arbacia* egg cells were stratified with high speed centrifugation. The cells were then imbedded in a 1% agar-sea water solution on a glass coverslip, placed on the stage of an inverted microscope and were sonated at 85,000 cps with a Mason horn. When the vibrating probe was applied to the cell surface in the region of the fat cap, a dispersion occurred. In the region of the hyaloplasm agitation can be easily induced and the fine structure can be disrupted. The nucleus was carried along by the agitation. The yolk region of the egg does not flow very easily and higher power levels of sonation were needed to produce agitation. A rare situation of a cluster of three cells were so positioned in the sound field that agitation was induced in all three. The white half of the egg was spun with the granular portion toward the probe. A red half spun on the outer edge of the sound field. Bodies tending to sink in a liquid behave differently than those which tend to rise. The axis of vibration in the pattern of the sound field and the interaction of the sound field with the cell produced these effects. A bubble of air in the sound field can be a sonic source for a neighboring cell. An analogy can be made to motion in a centrifuge. Further development is needed in acoustic streaming theory on Newtonian fluids to describe sonically induced motions in living cells.

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The cyclic elongation of stolons and uprights in the hydroid, Campanularia. CHARLES R. WYTTENBACH, SEARS CROWELL AND ROBERT L. SUDDITH.

The elongation of stolon and pedicel tips has been studied in *Campanularia flexuosa* colonies by recording their length against an ocular micrometer at 12-second intervals for periods of two hours each. Such "growth" occurs not as continuous gradual lengthening, but rather in defineable cycles or spurts. Each such cycle shows sequentially the following phases: (1) extension, (2) resting, (3) retraction, (4) resting, (5) re-extension to length in step (2), and (6) resting. Effects of the following upon the duration and nature of this cycle were studied: individual and genetic variability, temperature and isolation from the colony.

There is considerable variation among successive cycles in the same stolon as to the magnitude of extension (range 13–25 micra) and retraction (range 1–25 micra). Different stolons of the same genetic strain may differ somewhat in their *average* extension and *average* retraction per cycle for no apparent reason. A comparison of cycle time between two strains gives highly reproducible average values of 6.40 and 6.75 minutes at 20° C. Cycle time of the former strain at 16° C. is 8.80 minutes; otherwise the cycle is unaffected.

Pedicel elongation differs from stolon elongation by its more conservative extension phase (4–12 micra per cycle), virtual absence of retraction phase (0–3 micra per cycle), and a cycle time of just 5.80 minutes. That two cycle times may coexist in one colony rules out pressure from hydroplasmic streaming as the motive force, a conclusion reinforced by the observed persistence of pulsating growth in isolated 0.5-mm. stolon tips.

In both stolon and pedicel tips thickness of the epidermal cap varies throughout the elongation cycle, declining from maximum (50–70 micra) to minimum (about 20 micra) during execution of the extension phase, then thickening during subsequent retraction and resting phases. No support was seen for Berrill's (1949, 1961) observations that thickness of this cap is related to the rate of stolonic growth, or that annular growth of pedicels is due to alternating spurts of epidermal and gastrodermal growth.

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Cleavage delay and early quadripartition induced by β-mercaptoethanol. SHUHEI YUYAMA.

Eggs of *Arbacia punctulata* and *Lytechinus variegatus* were exposed to 0.1 *M* mercaptoethanol for short periods at different times after fertilization. In both the first and second cleavage cycle there is a phase starting from the "early streak stage," during which cleavage delay increases linearly (linear phase). During this phase the interval between the end of exposure and cleavage is constant, indicating that recovery takes a constant period of time. Eggs exposed to mercaptoethanol during puromycin-sensitive stages later exhibit a decreased sensitivity to puromycin. This suggests that the protein synthesis necessary for division continues during the mercaptoethanol block. During the linear phase, eggs are not sensitive to puromycin either before or after mercaptoethanol treatment, suggesting that additional protein synthesis is not required during the recovery period. A 15-minute exposure to puromycin, four minutes after fertilization, results in 30 minutes cleavage delay. When these puromycin-treated eggs are exposed briefly to mercaptoethanol, the linear phase is postponed 30 minutes. This indicates that the linear delay phase develops only after the synthesis of proteins required for division.

Lytechinus eggs, treated with mercaptoethanol before the first cleavage of controls, undergo quadripartition in the middle of the second cleavage cycle of controls. In these experiments the splitting of the two units of the mitotic centers may not be correlated directly with the normal mitotic schedule.

Eggs were cut into nucleate and anucleate halves and then fertilized. The androgenic halves undergo quadripartition after mercaptoethanol treatment, demonstrating that female pronuclei are not required for quadripartition. Since whole eggs and both types of half-eggs divide at the same time following mercaptoethanol treatment, the mercaptoethanol-induced cleavage delay appears to be independent of the delay characteristically caused by removing the female pronucleus.

Antagonists of DNA synthesis in epithelial cells of the dogfish (Mustelus canis) cornea. S. Zigman, S. Lerman and J. Tuttle.

Former studies showed that mechanical injury of dogfish cornea epithelium causes a rapid synthesis of new DNA (*i.e.*, H^3-thymidine uptake). These cells continue to incorporate thymidine into DNA during incubation in elasmobranch Ringer's solution. Thymidine incorporation is depressed markedly at lower temperatures or under decreased O_2 tension, indicating a metabolic dependence.

The effects of iododeoxyuridine (IUDR), of ultraviolet light and of γ-rays on the uptake of H^3-thymidine by the DNA of these cells were determined. Three epithelia per group were incubated for 5 hours at 20° C. under 95% O_2:5% CO_2 in 5 ml. of elasmobranch Ringer's medium containing 100 μC. (or 10^{-2} μM) of H^3-thymidine. After freezing on dry ice, the cells were homogenized in and washed with 5% TCA to remove all acid-soluble radioactivity. DNA was determined by the micro-indole procedure, and radioactivity was measured with a liquid scintillation counter. Ultraviolet light at 257 mμ was provided by a quartz immersion lamp, and γ-radiation by a Cs^{137} source.

Thymidine incorporation was decreased to 50% of normal by 10^{-3} μM IUDR; by two hours of UV exposure; or by 500 r of γ-rays. No inhibitor completely blocked thymidine uptake by DNA.

Possible mechanisms for these effects were explored. Using isotopic analysis, IUDR (10^{-4} μM) was found to replace 7% of DNA-thymidine (10^{-2} μM) during 5 hours of incubation. When equal levels of IUDR and thymidine (10^{-2} μM) were present, an 80% inhibition of thymidine uptake occurred, which indicates inhibition of an enzyme (thymidine kinase) rather than competition for sites of incorporation into DNA.

Ultracentrifugal and ultraviolet absorption analyses indicated that UV and γ-rays had broken DNA chains, thereby blocking DNA synthesis.

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DISTRIBUTION AND FUNCTION OF THE BRANCHIAL NERVE IN THE MUSSEL[1]

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The pumping of sea water by the mussel, *Mytilus edulis,* is accomplished primarily by the beating of cilia on the lateral epithelium of the gill filaments. The possibility that this activity is under the influence of the nervous system has been both suggested for its plausibility and dismissed for lack of evidence (Field, 1922; Gray, 1928; Lucas, 1931a, 1931b). More recently, however, work from this laboratory demonstrated that ciliary activity in the gill of *M. edulis* is depressed by transecting the branchial nerve (Aiello, 1960) and increased by electrical stimulation of the branchial nerve (Aiello and Guideri, 1964). In the latter paper it was mentioned that branches of the branchial nerve had been traced to the gill filaments. The present paper gives a detailed account of this work.

MATERIAL AND METHODS

Fresh mussels (*Mytilus edulis*) were purchased at a local fish market and kept in sea water at 5° C. until used. Isolated gill-nerve-ganglion preparations were made by removing one gill with its associated branchial nerve and visceral ganglion and a small piece of adductor muscle. Experiments were conducted in sea water at 22°–25° C., pH 7.7–7.9. Electrical stimulation (0.5 to 30 volts, 1 msec. biphasic pulse, 10 pulses per second for 5 to 300 seconds) was supplied by a Grass Model S4 stimulator and delivered through a pair of tungsten wires, one inserted into the adductor muscle and the other placed in the desired position on the ganglion, nerve or gill. The rate of beating of lateral cilia was determined by synchronization with stroboscopic light as described earlier (Aiello, 1960).

Histological sections were made from animals prepared *in toto* after removing the shell. The following preparations were made: fixed in 0.1% osmic acid in sea water and left unstained; fixed in 10% formalin in sea water and left unstained or stained with Harris hematoxylin and eosin; fixed in formalin-acetate and stained with dimethylaminobenzaldehyde (Glenner and Lillie, 1957); gold impregnation (Cole, 1946); silver impregnation (Rowell, 1963); silver impregnation after

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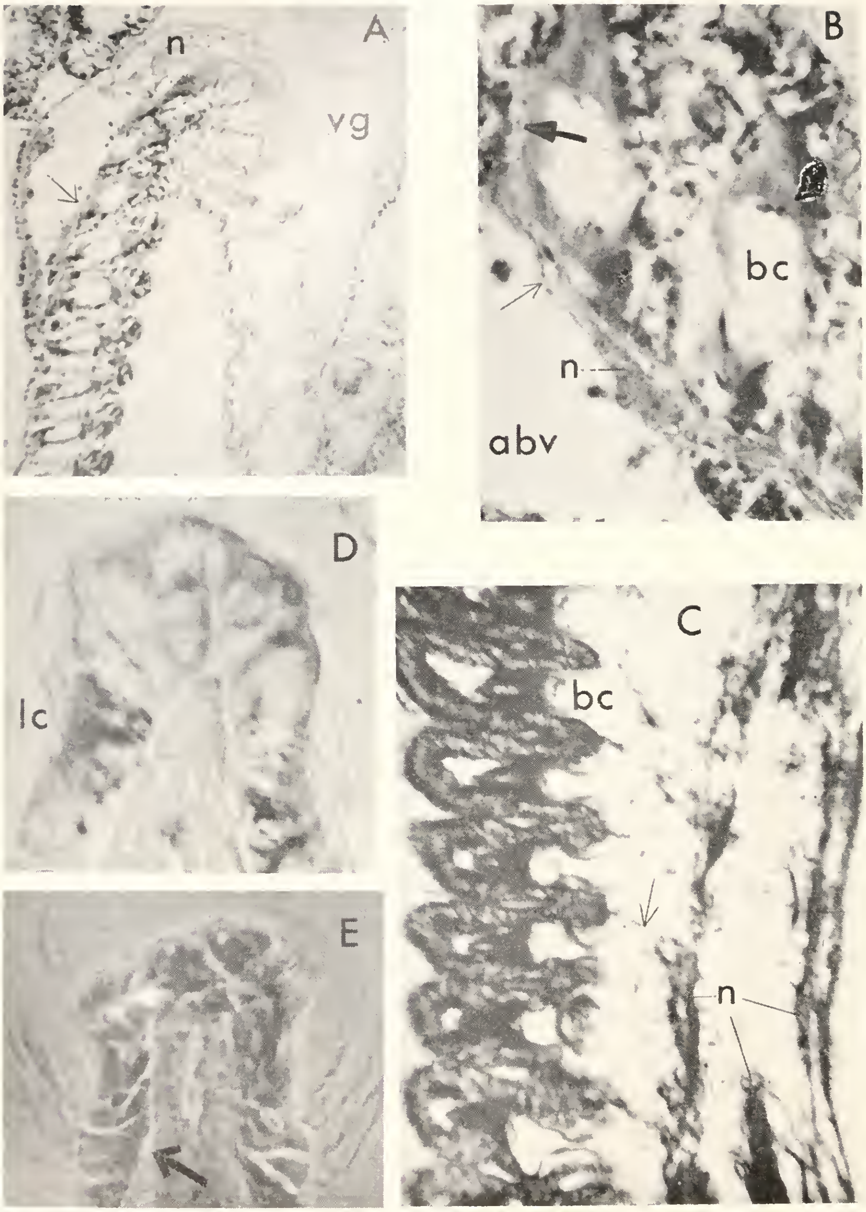

Fig. 1. Gill of *Mytilus edulis.* A. Cross-section of gill axis from whole animal fixed in osmium tetroxide; hematoxylin and eosin; 10 μ thick; 87 ×; shows visceral ganglion (vg) and branches of the branchial nerve (n); arrow indicates a nerve fiber entering a filament. B. Same preparation as A; 280 ×; small arrow indicates same nerve fiber as in A; large arrow indicates a continuation of the same fiber under the epithelium; nerve bundle (n) is inside the afferent branchial vein (abv) but separated from the blood channel (bc) by the abfrontal end of the filament. C. Horizontal section through the gill at about the level of the nerves indicated by n in B above; Cole gold chloride stain; 10 μ thick; 332 ×; one of a group of nerve bundles (n)

treatment with copper sulfate (Betchaku, 1960). Sections were cut at 5 to 40 μ thickness in the following planes relative to the intact animal and gill axis: cross, giving longitudinal sections of the gill filaments; horizontal (frontal), giving cross-sections of the gill filaments; and sagittal, giving frontal sections of the gill filaments.

Whole mounts and teased preparations were left unstained or treated with gold, hematoxylin and eosin, or 0.2% methylene blue in sea water. Whole living animals were also studied after injecting the visceral mass or the afferent branchial vein with 0.2% methylene blue in sea water or Pelikan brand India ink diluted with nine volumes of sea water.

Observations were made under bright field, phase contrast and epi-illumination, depending on the specimen.

RESULTS

Distribution of the branchial nerve

Careful observation, aided by fine dissection, of the ventral aspect of the soft parts of the mussel under a dissecting microscope at 30 × magnification confirms in general the description of the branchial nerve given by Field (1922; p. 172): "The branchial nerve arises from the outer posterior side of the visceral ganglion and runs obliquely downward and backward to the base of the gills, which it follows to the posterior extremity. Throughout its course, but more so at its beginning, it gives off a great number of very fine fibrils that run in a mass anteriorly along the axis of the gills." In addition a great number of fibers arise directly from the visceral ganglion, and these can be seen clearly silhouetted against the underlying blood vessels when the latter are filled with India ink injected in the afferent branchial vein. The anteriorly directed fibers do not continue to run in a mass but distribute themselves as bundles which run in close association with the afferent branchial vein.

Although the visceral ganglion and branchial nerve lie on the median side of the gill axis, some branches of the branchial nerve pass through the supporting tissue above the blood vessel to supply the lateral side of the gill. This distribution is evident in sections of fixed material (Fig. 1 A) and in the published works of Lucas (1931b, his Plate I, Fig. 3) and Field (1922, his Figs. 147 and 153), although Field did not identify branches of the branchial nerve as such. In our material each individual bundle remained fairly intact until it reached a particular part of the gill axis, whereupon it distributed its fibers to a series of adjacent filaments. Such a point is indicated by the arrow in Figure 1 A. This nerve bundle lies within the afferent branchial vein and sends fibers into the filament between the chitinous rod and the ciliated epithelium. This is seen more clearly in Figure 1 B which is an enlargement of the area around the arrow in Figure 1 A. The heavy arrow in Figure 1 B points to a continuation of a nerve fiber under the

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sends a few fibers, marked by the arrow, to a filament; the blood channel (bc) opens directly into the afferent branchial vein. D. Cross-section of a single filament; Betchaku copper-silver stain; 10 μ thick, 921 ×; lateral cells (lc) show intense staining; nerve fibers appear in cross-section as lighter-stained dots at base of lateral cells. E. Cross-section of single filament; Glenner and Lillie DMAB-fuchsin stain; 5 μ thick; 883 ×; arrow indicates two nerve fibers at the base of the lateral cells.

ciliated epithelium, which at this level is not differentiated into special tracts. The lining of the blood vessel seems to be discontinuous in this region and the nerve labeled *n* in Figure 1 B distributes itself to the adjacent filaments. Ventral to this region a continuous layer of connective tissue separates the other nerve bundle from the filaments. This bundle, which appears much darker and lies below and to the right of the one marked *n* in Figure 1 B, was followed anteriorly in serial sections for about 0.5 mm. and found to move up toward the gill axis and enter the filaments in a similar manner. A horizontal section through this part of the gill confirms these observations (Fig. 1 C). In all preparations nerve fibers were detected in cross-sections of gill filaments as groups of circular or oval structures lying at the base of the lateral cells (Fig. 1 E). They were sometimes associated with branching structures seen only with gold or silver staining (Fig. 1 D). Single fibers seem to be present under other parts of the epithelium but because they do not occur there in groups they could not be traced with certainty back to the branchial nerve. Fibers under the lateral cells appear to course the length of the filament; usually three or four individual fibers could be counted. Branching was most frequently observed immediately after the nerve first entered the filament. Although one would expect to see individual fibers as small dots in every cross-section, we could find silver-staining structures under the lateral cells in only about 20% of the sections. In longitudinal sections of the filaments (frontal sections of the gill) in which the cut happened to be made through the base of the lateral cells, one would expect to see the fiber as a thin black line underneath the lateral cells. Actually, fibers were easily identifiable as such only where they first entered the filaments. Further along in the filament we most frequently saw a string of granules. Our interpretation of this pattern is that they are vesicles of 0.25–0.4 μ diameter along a fiber whose diameter is about the same as the resolving power of the microscope (0.21 μ). The fact that this structure is not merely a basement membrane was especially clear in those sections in which the cells were torn off the chitinous rod, leaving the nerve fibers in a clear space.

Fine structure and staining affinities of the branchial nerve

Grossly, the branchial nerve and visceral ganglion appear opaque white. After osmium fixation, nerve fibers, connective tissue fibers, cilia and many other structures appear black but there is no evidence of a myelin sheath comparable to that of vertebrate myelinated nerve. After formalin fixation and staining with Harris hematoxylin and eosin, the nerve cell cytoplasm stains faint blue, the nucleus darker blue, the nucleolus lighter blue and the fibers stain hardly at all. In both preparations the nerve fiber appears to be surrounded by an unstained space and a limiting membrane. After staining for indoles with dimethylaminobenzaldehyde the ganglion and the nerve appear faint blue. A blue tinge is also evident in nerve fibers under the lateral epithelium cells which themselves are deeper blue or blue-gray, especially in their basal half. Large blue granules are also seen in areas having no identifiable innervation.

In unstained formalin sections the nerves are colorless. Under phase contrast a nerve fiber in cross-section appears as a dark spot in a clear circle with a limiting membrane. Methylene blue stained the nerve after about one hour but was not found to be very useful because it stained most other cells more intensely. Individual fibers were not visibly stained. With gold the fibers in the nerve bundles appeared brown or black, depending on the depth of staining. In some sections stained with silver after copper treatment, the fibers under the lateral cells and associated structures lying in or between the basal half of these cells were stained black, in clear distinction to other structures which were stained various depths of brown. A second black-staining structure was situated around the nucleus of the lateral cell. It was not connected to any nerve and may have been Golgi material.

Conduction pathways

Electrical stimulation of various parts of the ganglion and of various nerve bundles in the gill axis caused cilio-excitation of particular filaments. In these experiments the electrode was placed on the surface of the ganglion or inserted into one of the bundles in the supporting tissue of the gill axis. The stimulus was applied and the voltage slowly increased until some part of the gill was found to have rapidly beating cilia. The stimulus was then discontinued and the ciliary beating rate decreased. This was repeated a few times to make sure that the applied stimulus was the cause of the cilio-excitation in that area of the gill. Using the lowest effective voltage, cilia on only a few filaments within 2 or 3 mm. of each other were excited. By placing the electrode on different parts of the ganglion it was possible to affect different small areas of the gill. Stimulation of branches of the branchial nerve affected only filaments directly below or anterior to the point of stimulation. When the reflected (ascending) lamella was separated from the main (descending) lamella at the ventral end but left connected by the interlamellar blood vessels, stimulation of the nerve to that filament resulted in excitation of the cilia on the main lamella but not those on the reflected lamella. This indicates that cilio-excitatory fibers do not pass over to the reflected lamella through this connection but instead continue down the main lamella and up the reflected lamella under the lateral epithelium. These interlamellar connections must have their own nerve supply which is distinct from the cilio-excitatory nerves, because during these experiments contraction of muscles lining the interlamellar blood vessels usually, but not always, occurred in those filaments showing cilio-excitation. Generally, 20 to 30 volts were required to elicit muscle contraction, whereas cilio-excitation could be obtained with 5 to 10 volts. That this muscle contraction is not due merely to current spread is indicated by the fact that the electrodes must be placed in just the right position at the base of the gill to get the effect. Stimulation of the visceral ganglion always caused a gross movement of the entire gill of a few millimeters, due to contraction of these muscles.

Further evidence that the sub-epithelial nerve is the only pathway for cilio-excitation is provided by the observation that damage to the surface of the lateral cells prevents their cilia from beating but does not prevent nerve stimulation from simultaneously activating the cilia on lateral cells both central and peripheral to the damaged area. However, damage which is deep enough to remove all cellular material, leaving a bare strip of chitinous rod, does prevent nerve stimulation from affecting lateral cilia peripheral to the damage. These cells are still stimulated by exogenous 5-hydroxytryptamine.

DISCUSSION

Distribution and function of the branchial nerve

The data presented above give the morphological basis for the nervous control of ciliary activity and movements of the gill filaments. The latter system was described by Setna (1930) in *Pecten* sp., in which he found fibers in both the abfrontal and frontal region of the gill. Those in the abfrontal region innervated the muscles of the interlamellar connections. Those in the frontal region were not traced to terminations and the question of ciliary control was not discussed. In our work we could not trace fibers to the interlamellar connections but could occasionally activate them independently of cilia, suggesting a separate nerve. The function of these muscles is not known but it has been observed that in addition to moving the filament slightly, they cause accordion-like shortening of the interlamellar blood vessel. This causes the blood to shoot up the filament and, even though it washes back again during relaxation, the to-and-fro movement might increase the efficiency of an otherwise very sluggish branchial circulation. The same rhythmic movement occurs spontaneously in the intact animal.

Nervous stimulation also influences the frontal and latero-frontal cilia. These are concerned mainly with feeding, and would presumably be most active when the number of food particles impinging on the frontal surface of the gill was greatest. Because this factor is not directly related to the rate of lateral ciliary beating it would not necessarily be to the animal's advantage to activate all cilia concurrently. In our experience stimulation of the lateral cilia was accompanied by an apparent increase in the beating of the lateral and latero-frontal cilia, but since the rate of these cilia was not measured we cannot be sure of quantitative relationships. When the branchial nerve is cut, the lateral cilia stop first, followed by the latero-frontal cilia (Aiello, 1960). The frontal cilia continue to beat, but more slowly. There is, therefore, no evidence yet for the independent control of different tracts of ciliated epithelium on the same filament.

There is morphological and physiological evidence for independent control of filaments. About 10 adjacent filaments are innervated by fibers from one bundle. Possible advantages to the mussel are: the flow of water over particular respiratory plicae could be adjusted; some filaments could pump water while others rested; frontal cilia on those filaments being struck by food particles could be selectively accelerated. Whether or not the animal does these things is not known but it has been observed by ourselves and others (Babak, 1913; Lucas, 1931a) that lateral cilia on various filaments periodically stop for a few seconds and then start beating again. Particle transport is also influenced by mechanical factors (Gosselin and O'Hare, 1961) and one of these, interfilamentar spacing, is under nervous control in lamellibranchs. This was reported for *Ostrea virginica* (Jørgensen, 1955) but the problem has not been studied in *Mytilus* sp. Our experiment, in which low voltage stimulation of particular spots on the surface of the visceral ganglion caused cilio-excitation on individual filaments, indicates that the distribution of the postganglionic filbers permits such discrete control. Whether or not presynaptic arrangements permit such control in nature was not determined. The question of intra-ganglionic connections in *M. edulis* visceral ganglion was studied by Rawitz (1887), who concluded that fibers from the peripherally placed cell bodies form a nerve net in the center of the ganglion. This arrangement would be likely to give mass discharges of cilio-excitation. Fortuyn (1920) came to a contrary conclusion, which is more compatible with our physiological data, namely, that the central mass of fibers constitutes a neuropile with few if any functional connections. He also described the localization within the ganglion of association neurons. Freidenfelt (1897) came to a similar conclusion regarding fibers in the central mass of the visceral ganglion of *Anodonta* sp. In addition, Freidenfelt described association neurons at the junction of the posterior pallial and branchial nerves near their emergence from the visceral ganglion, and pointed out their close association with the overlying osphradium. He postulated that the osphradium and the root of the branchial nerve constituted an independent sensory-motor center. He was referring to motor fibers to smooth muscle in the subfilamentar structure of *Anodonta* gill, but the same reasoning could apply to cilio-excitatory fibers to the epithelium in *M. edulis*. There is no evidence in the literature or in our data for sensory fibers in the gill filaments.

Innervation of the ciliated cell

We are uncertain of the detailed structure of the innervation apparatus. We only observe that the nerve fibers run between the base of the ciliated cells and the supporting chitinous rod. With silver stain, there seems to be a basket-like structure in the basal half of the lateral cell which appears to be either connected to or closely apposed to the underlying fiber. In view of the relationship between endogenous 5-hydroxytryptamine and cilio-excitation through branchial nerve stimulation (Aiello, 1965), it is interesting that the lower half of the lateral cell and the branchial nerve throughout its length stain faint blue with dimethylaminobenzaldehyde, thereby suggesting the presence of 5-hydroxytryptamine. Presumably, nerve impulses release 5-hydroxytryptamine from either the nerve fiber or the ciliated cell and this in turn stimulates ciliary activity.

We thank Miss Linda Gottfried for technical assistance.

SUMMARY

1. Using standard dissecting and histological techniques, branchial nerve fibers have been traced from the visceral ganglion to the ciliated epithelium of the gill in the mussel, *Mytilus edulis*.

2. The pattern of activation of cilia obtained by electrical stimulation of the visceral ganglion and branchial nerve indicates that individual filaments or small groups of adjacent filaments are independently innervated, allowing for discrete control of ciliary activity on different parts of the gill.

3. The relationship of this innervation to the known functions of the gill is discussed.

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INHIBITION OF BLOOD DIGESTION AND OOCYTE GROWTH IN AEDES AEGYPTI BY 5-FLUOROURACIL

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5-Fluorouracil (FU) inhibits oviposition in house flies, screw-worm flies and fruit flies (Smith *et al.,* 1964). This compound sterilizes only females (Crystal, 1963) and its sterilizing effect is temporary (Painter and Kilgore, 1964). Only the final effect of FU-treatment, *i.e.,* the inhibition of oviposition, has been observed in these studies. It is not known at what stage egg maturation was arrested, and whether any yolk was formed.

The morphological and physiological changes which occur in the ovaries and also in extraovarian tissues during egg maturation in *Aedes aegypti* have been extensively studied, and summarized in Christophers' (1960) and Clements' (1963) monographs. In this insect egg maturation is initiated by a blood meal; this fact makes it possible to recognize the sequence of events leading to egg formation, and their timing in relation to the blood meal.

Before the blood meal the ovaries are in a "resting stage." Shortly after gorging, a hormonal factor is released which initiates oocyte growth. Four hours after the blood meal, yolk deposition is already evident in the oocyte (Laurence and Roshdy, 1963). Yolk protein uptake by the oocyte is most active seven hours after the blood meal; yolk synthesis and storage are essentially completed 25 hours after the meal (Roth and Porter, 1964).

Very little proteolytic activity can be detected in the midgut of *Aedes aegypti* before the blood meal. Midgut protease activity rapidly rises in the first few hours and reaches its peak 18–24 hours after engorgement (Fisk and Shambaugh, 1952). Two days after a blood meal (at 28° C.) blood digestion is completed and the ovaries contain mature eggs.

This paper describes the effect of FU on the rate of oocyte growth and blood digestion in *Aedes aegypti* females.

MATERIALS AND METHODS

Aedes aegypti mosquitoes ("Ness-Ziona" strain, kept at this laboratory since 1950) were reared under standard laboratory conditions at 28° C. FU (Hoffmann La-Roche) was given in 5% sucrose for two days, or in sheep blood (containing 0.25% sodium citrate) through Silver-light membranes (Galun *et al.,* 1963). Adult mosquitoes, before and after treatment, were kept on 5% sucrose at 28° C.

In oviposition experiments single engorged females were confined in gauze-covered 170-ml. glass jars, containing a small aluminum cup filled with water and lined with a strip of filter paper (these females were fed on honey). The day of the oviposition was noted, and the eggs were kept for a further three days in moisture at 28° C. and then dried. The number of eggs and the percentage of hatching were determined for each batch. Hatching was stimulated by lowering the oxygen tension in the water containing the eggs (Clements, 1963). Each batch of eggs was immersed in tap water in a 10-ml. beaker; the beakers were placed in a desiccator, and the pressure was reduced to 70 mm. Hg. for 10–20 minutes to remove the air from the water.

At daily intervals after the blood meal the females were dissected in 0.85% NaCl and the size of the ovarian follicles was measured under a binocular microscope. The ovarian development was divided into eight stages, based on Christophers' classification (1960) as modified by Clements (1963). Stage 1 is the resting stage, corresponding to Clements' stage IIa, stage 2 corresponds to IIb. We have subdivided stage III into three stages (3–5), according to the amount of yolk formed. Our stages 6–8 correspond to Clements' (1963) stages IVa, IVb and V, respectively. The division into eight stages enabled us to calculate the average stage of the ovaries at various intervals after a blood meal.

Stage 1. No yolk visible under low-power (100 ×) magnification (resting stage).

Stage 2. Yolk granules visible in the oocyte under a binocular (ovaries have passed the resting stage).

Stage 3. The oocyte (20–50 microns in length) occupies $\frac{1}{4}$–$\frac{1}{3}$ of the follicle.

Stage 4. The oocyte (51–100 microns in length) occupies $\frac{2}{5}$–$\frac{3}{4}$ of the follicle.

Stage 5. The oocyte (101–150 microns in length) occupies about $\frac{4}{5}$ of the follicle.

Stage 6. The oocyte (151–250 microns in length) occupies about $\frac{9}{10}$ of the follicle.

Stage 7. The follicles (251–350 microns in length) assume the shape of the mature eggs.

Stage 8. The ovary contains mature eggs with visible chorionic structures.

The stages of blood digestion (presented schematically by Stohler, 1957) can be easily observed under low magnification. The color of the ingested blood is bright red in freshly engorged females. Digestion begins at the periphery within the gut's lumen, and can be recognized by the brownish color around the periphery several hours after the meal (Stohler's stages A and B). Blood digestion proceeds from the periphery inwards. About one day after engorging, the blood mass is brown except for the center (Stage C). When the color of the blood mass is brown throughout, digestion is completed and elimination begins. The size of the blood mass decreases and the midgut walls become again folded (stage D).

Blood-fed females were dissected in 0.85% NaCl at daily intervals after the blood meal, and 12–30 midguts from each group were kept in a small glass vial at −18° C. until needed (no decrease of protease activity was observed after two months' storage at −18° C.). The protease activity was tested by a modified procedure of Tomarelli *et al.* (1949), using azoalbumin as substrate. The midguts were homogenized in a glass homogenizer in cold 0.1 *M* sodium phosphate buffer, pH 7.8, containing 1:20,000 thiomersalate (0.1 ml. buffer for each gut). Azoalbumin was dissolved in the same buffer, 25 mg. per ml. The incubation mixture consisted of 0.3 ml. 0.1 *M* buffer, pH 7.8 (which was shown to be optimal by Fisk, 1950), 0.4 ml. substrate solution (containing 10 mg. azoalbumin) and 0.3 ml. midgut homogenate (equivalent to three midguts). All experiments were run in duplicate. The tubes were incubated for 30 minutes at 39° ±0.1° C. The reaction was terminated by the addition of 3.0 ml. 6% trichloroacetic acid. The precipitated protein was separated by centrifuging and filtering. Three ml. of the supernatant from each tube were mixed with 3.0 ml. 0.5 N NaOH, and optical densities were determined at 440 millimicrons. Protease activity was measured as mg. azoalbumin digested by three midguts in 30 minutes.

RESULTS

Effect of feeding FU in the blood meal on oviposition

Six-day-old mated females were given FU in sheep blood through membranes. The FU in the blood meal did not interfere with normal feeding, but all the engorged females died within one day when the final concentration of FU was 0.5%. With 0.25% FU 30% mortality occurred; lower concentrations did not affect the viability. The minimal dose which inhibited oviposition was 0.025% FU (Table I).

TABLE I

Effect of feeding FU in the blood meal on oviposition

% FU	Oviposition after 1st blood meal (Sheep blood mixed with 0.85% NaCl)			Oviposition after 2nd blood meal (on rat)		
	No. of females ovipositing	Eggs per ovipositing female*	% inhibition of oviposition**	No. of females ovipositing	Eggs per ovipositing female	% inhibition of oviposition
none	36/47	51.2	—	54/55	101.0	—
0.005	7/14	45.7	41.7			
0.010	0/18	9	98.7			
0.025	0/20	—	100			
0.05	0/20		100	24/43	32.2	81.8
0.25	0/19		100	4/28	35.0	95.0

* The percentage of hatching was over 80% in all batches of eggs.

$$^{**}\ \frac{\text{Eggs per female in control—eggs per female in treated}}{\text{Eggs per female in control}} \times 100.$$

Since the amount of blood taken up by a fully engorged female is about 2 mg., the LD_{50} is between 5 and 10 µg. FU, and the dose which causes 50% inhibition of oviposition between 0.1 and 0.2 µg. FU per female. FU-treatment did not affect the viability of the eggs laid; 83–89% of the eggs laid by the treated females hatched, as compared with 91% in the controls.

The effect of FU on oviposition was not permanent. More than half of the females which ingested 0.05% (two times the minimal dose which inhibited oviposition) and over 10% of those which took 0.25% (10 times the minimal dose inhibiting oviposition) in their first blood meal, oviposited viable eggs after a second blood meal (on a rat), which was given one week after the FU-treatment (Table I).

The blood meal, consisting of one part of 0.85% NaCl to three parts of sheep blood, was nutritionally suboptimal. About a quarter of the engorged controls did not lay any eggs, and the average number of eggs laid was only about half of that following a blood meal on a rat. In order to study the effect of FU under conditions of optimal nutrition, it was more convenient to treat the females first and then feed them on a rat.

Effect of feeding FU in sugar solution on oviposition

One-day-old mosquitoes were fed FU in 5% sucrose for two days, kept on sugar solution for two more days, and then offered a rat. The average weight of the blood meal taken by the females treated with 0.05% FU was not less than that taken by the controls (2.3 mg. and 2.16 mg., respectively). Egg production in mosquitoes treated with 0.05% FU two days before their blood meal was greatly inhibited (see Table II. The results with 0.05% FU were pooled from 5 experiments, each consisting of 10–12 engorged females; in two experiments no oviposition occurred).

TABLE II

Effect of feeding FU in sugar solution on oviposition
(FU in 5% sucrose for two days to one-day-old mosquitoes, blood meals on rat)

Time of first blood meal	% FU	Oviposition after 1st blood meal			Oviposition after 2nd blood meal		
		No. of females ovipositing	Eggs per ovipositing female*	% inhibition of oviposition**	No. of females ovipositing	Eggs per ovipositing female	% inhibition of oviposition
Rat two days after removal of FU	none	49/50	93.1	—	23/24	93.7	—
	0.0125	18/24	80.7	33.6	13/13	87.6	2.4
	0.025	15/24	63.6	57.3	14/14	75.9	15.4
	0.05	5/54	54.2	94.5	16/28	56.5	64.0
Rat 14 days after FU	none	14/14	102.4	—			
	0.05	4/9	11.5	94.7			

* The percentage of hatching was over 80% in all batches of eggs.

** $$\frac{\text{Eggs per female in control—eggs per female in treated}}{\text{Eggs per female in control}} \times 100.$$

When the interval between FU-treatment (0.05%) and the blood meal was prolonged from two to 14 days, a greater percentage of females was able to lay eggs, though there was little recovery in terms of the average number of eggs per female. The recovery of the females treated with 0.05% FU was more obvious after the second blood meal (Table II).

The eggs laid by the females treated with FU in sugar solution also hatched normally (80–88%). Although FU in sugar solution was given to both sexes, it evidently affected only the oviposition, but had no effect on the viability of the eggs.

Table II gives only the number of eggs per female after the first and the second blood meal. However, FU also affected the time of oviposition. The controls that fed on rats started ovipositing two days after feeding, and all of them completed oviposition within three days after the blood meal. Females that were treated with 0.0125% and 0.025% FU started ovipositing 4–5 days after feeding on blood; those treated with 0.05% FU oviposited 5–7 days after their first blood meal. After the second blood meal oviposition was also later than in the controls, but only in the group treated with 0.05% FU. Dissection of the mosquitoes showed that the delay in oviposition in the treated females was due to slower maturation of the oocytes.

Effect of FU on the rate of oocyte growth

The ovaries of gravid females were examined daily after feeding on a rat, until oviposition occurred, and the stage of oocyte development was determined. (Twenty to 80 females were dissected daily in each group.) The stages of ovarian development were divided into 8 stages as described in Materials and Methods. The average stage of the ovaries on each day after the blood meal, and the percentage of females containing mature eggs, are given in Figure 1.

The rate of oocyte growth and the percentage of females developing mature eggs was proportional to the amount of FU given and the time interval between FU-treatment and the blood meal. When five-day-old mosquitoes were fed 0.05% FU for two days immediately before their blood meal oocyte growth was completely inhibited beyond stage 5. The ovaries of most of the females remained in stages 2 and 3, and only 2% of all dissected females had reached stage 5. When the first blood meal was given two days after 0.05% FU, a few females were able to develop mature eggs (Fig. 1).

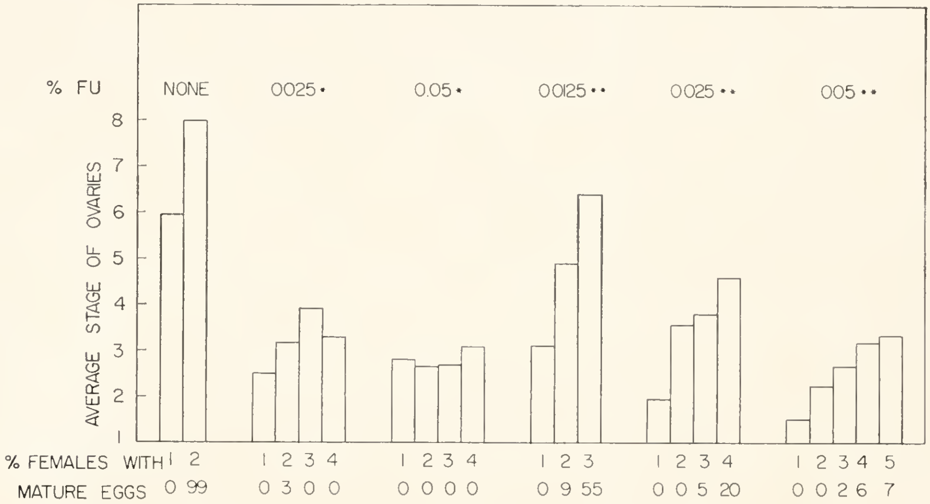

FIGURE 1. Effect of FU on the rate of ovarian development (FU in 5% sucrose for two days, blood meals on rats). The numbers below the columns indicate the day after the blood meal. * Blood meal immediately after FU. ** Blood meal two days after FU.

The ovaries of the treated females which were in stages 2–4 could not be distinguished from normal ovaries in a comparable stage of development. However, some of the ovaries of treated mosquitoes, which developed beyond stage 4, showed irregular development (irrespective of the concentration of FU). Normally all the growing follicles in mosquito ovaries are of uniform size. In some of the FU-treated females the rate of growth of individual follicles varied. A small number of follicles (often only one or a few per ovary) had reached stages 6–8, whereas the remainder was of a size typical of stage 4 ovaries. This asynchronous development affected both ovaries. The stage of ovarian development assigned to asynchronous ovaries was that of the largest follicle present (for the computation of the averages in Figure 1). Ovaries were considered as stage 8 even when only a few follicles developed mature eggs.

A second blood meal was offered 8 days after the first (10 days after removal of FU). The rate of egg maturation (and also the number of eggs produced, see Table II) in the females treated with the lower concentrations of FU was almost equal to that of the controls. Two days after the blood meal 96.2% and 87.5% of the females treated with 0.0125% and 0.025% FU, respectively, contained mature eggs. In females treated with 0.05% maturation of eggs was slower. Only 33% contained mature eggs two days after the second blood meal; the ovaries of the rest were in stages 4–6.

Effect of FU on the rate of blood digestion

In untreated females blood digestion at 28° C. is completed about 36 hours after feeding on a rat, and defecation begins. Forty-two hours after feeding 66% of the females had empty midguts; 33% contained small blood residues (a quarter of the original amount). Of 152 females dissected 48 hours after the blood meal, 23% contained blood residues in the midgut or in the hindgut (Fig. 2). No traces of the blood meal were evident in untreated females 54 hours after engorging.

The females treated with FU immediately before the blood meal, or two days before the blood meal, retained the blood in their midguts much longer than the controls (Fig. 2). The delay in defecation was proportional to the amount of FU given.

The time of 50% digestion is the time in hours after a blood meal, when 50% of the females had completely emptied their guts (Fig. 2). The point of 50% digestion time was obtained by interpolation between the two adjacent days (when less or more than 50% of the midguts were empty) on log probability paper. The results in Figure 2 indicate that the period of blood digestion in females treated with 0.05% FU is more than twice that in the controls.

In our oviposition experiments we measured the number of eggs per blood meal. If the mosquitoes had free access to blood meals, oviposition could be measured per unit of time. The controls could thus have taken more blood meals and accomplished more gonotrophic cycles than the FU-treated females. The inhibition of oviposition by the lower concentrations of FU would be more pronounced under these conditions than in our experiments (Table II).

All the females whose ovaries reached stage 8 had completed blood digestion by that time. The females whose ovaries remained undeveloped retained the blood longer, but eventually emptied their midguts without developing mature eggs.

The rate of blood digestion returned to normal after the second blood meal in the females treated with 0.025% and 0.025% FU (in these groups maturation of eggs also proceeded at a normal rate). Recovery after the second blood meal was slower in the females treated with 0.05%.

The females whose ovaries had reached stage 8 two days after the second blood meal had also completed blood digestion by that time (see above). The midguts of 66% of the females whose ovaries had not developed beyond stage 6 were still full two days after feeding (*i.e.,* blood digestion and ovarian development were in the stage reached by the controls one day earlier).

The delay in blood digestion in the FU-treated females was evident even before defecation started. The fact that blood digestion was greatly retarded in the treated females could be observed by the color of the blood mass one day after feeding. The whole blood mass in the untreated females was brown, whereas the blood mass in the females treated with 0.05% was bright red except for a thin brownish layer at the periphery. Blood digestion (as estimated by the color of the blood mass one day after feeding) had proceeded further in the females treated with 0.0125% and 0.025% FU, but did not reach the stage of the controls. Since the changes in the color of the blood mass indicate the extent of proteolytic activity, the midgut protease of the FU-treated females was examined *in vitro.*

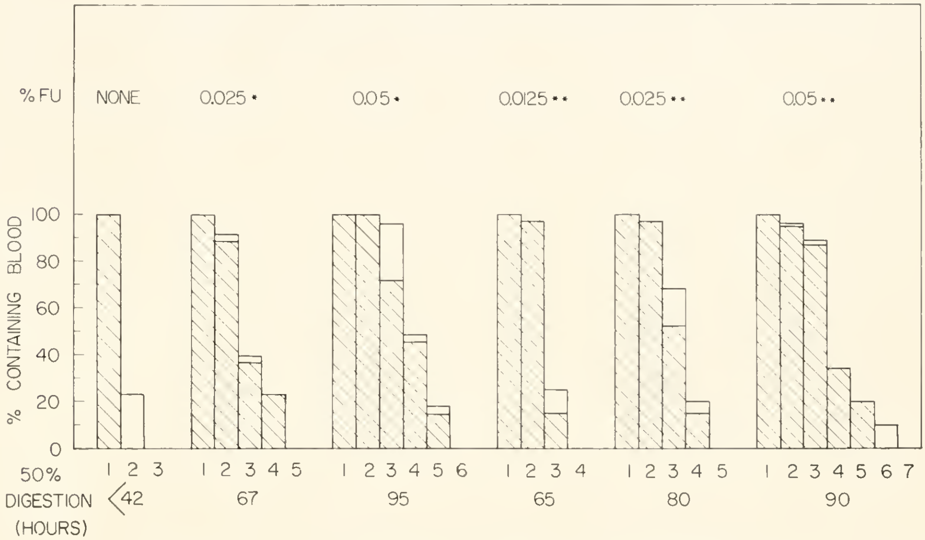

FIGURE 2. Effect of FU on the rate of blood digestion (FU in 5% sucrose for two days, blood meals on rats). The numbers below the columns indicate the day after the blood meal. Shaded columns indicate full midguts; empty columns blood residues. * Blood meal immediately after FU. ** Blood meal two days after FU.

Effect of FU on midgut protease

The protease activity was measured as mg. azoalbumin released by a homogenate of three midguts (including the content of the lumen) after 30 minutes' incubation at 39° C. The reaction proceeded linearly up to about 30 minutes; after that the rate of the reaction decreased gradually. There was little proteolytic activity after three hours. No protease activity could be detected by our method in untreated midguts dissected immediately after engorging. The average protease activity 4–5 hours after feeding was about 0.15 mg. per three midguts. Protease activity of untreated midguts reached its peak 18–24 hours after feeding. At that time the average protease activity was 0.874 ± 0.135 (standard deviation) mg. azoalbumin released by three midguts in 30 minutes at 39° C. (13 experiments). The protease activity of untreated midguts was low 48 hours after blood meal (0.028 mg., average from four experiments), but only 25% of the midguts contained visible blood residues; the rest were empty.

The protease activity of homogenates prepared from a mixture of empty and blood-containing midguts is due to the blood-containing midguts. No protease could be detected by our method in midguts which have recently been emptied (42 hours after feeding). The average protease activity of midguts which still contained visible traces of the blood meal 42–46 hours after feeding was 0.11 mg. azoalbumin per three midguts.

TABLE III

Midgut protease activity in FU-treated females. (FU in 5% sucrose for two days)

Treatment	% FU	Day after blood meal	% with blood-filled midguts	Average protease activity*
FU given to 5-day-old mosquitoes. Blood meal immediately after removal of FU	none	1	100	0.77
	0.025	1	100	0.15
		2	89	0.34
		3	36	0.27
		4	23	0.21
	0.05	1	100	0.15
		2	100	0.22
		3	72	0.12
		4	45.5	0.15
FU given to one-day-old mosquitoes. Blood meal two days after removal of FU	none	1	100	0.86
	0.0125	1	100	0.33
		2	100	0.61
	0.025	1	100	0.20
		2	95	0.43
		3	52.5	0.30
	0.05	1	100	0.19
		2	95	0.26
		3	75	0.41

* Mg. azoalbumin per three midguts, incubation 30 minutes at 39° C. Only blood-filled midguts, 12–30 for each test, were taken.

Fisk and Shambaugh (1952) found a small amount of "residual" protease in unfed and sugar-fed mosquitoes (after 5 hours of incubation). This amount was too small to be detected by our technique. Our results regarding the amount of protease at various times after a blood meal in untreated females are similar to those obtained by Fisk and Shambaugh, if adjusted to the differences in the time of incubation. In their experiments considerable protease activity was found 48 hours after feeding, but they kept the females at a lower temperature. Blood digestion in *Aedes aegypti* is known to be slower at lower temperatures (Stohler, 1957).

The midgut proteolytic activity of FU-treated females is given in Table III. Only midguts that were fully distended with blood were taken for the protease assay (in order to make sure that we measured the protease activity before the peak, or near to it, and not the residual protease after digestion had been completed). The percentage of females containing blood-filled midguts on each day after feeding is given in Table III. The protease activity of the controls two days after feeding is not included, since none of the controls had blood-filled midguts by that time.

The inhibition of the protease activity in the FU-treated females was most obvious 24 hours after blood meal, when the protease activity in the controls was at its peak. In the treated females protease activity was higher two days after feeding than on the preceding day. Though the peak protease activity in the treated females occurred 1–2 days later than in the controls, the amount of protease in FU-treated females never reached the peak values of the controls. Protease inhibition was higher when the blood meal was given immediately after the FU treatment, and it was proportional to the amount of FU (Table III).

TABLE IV

Recovery of midgut proteolytic activity after FU-treatment (0.05% FU in 5% sucrose given to one-day-old mosquitoes for two days. Blood meals on rat)

Time of blood meal	Day after blood meal	Average proteolytic activity*	
		Treated	Controls
First meal two days after FU	1	0.14	1.07
	2	0.23	—
	3	0.23	—
First meal 7 days after FU	1	0.10	0.85
	2	0.33	—
First meal 14 days after FU	1	0.36	0.84
Second meal 10 days after FU	1	0.64	0.84

* Mg. azoalbumin per three blood-filled guts, incubation 30 minutes at 39° C.; 12–20 midguts were taken for each test.

In order to test whether inhibition of protease by FU was caused by the direct inactivation of the enzyme, the effect of FU was tested *in vitro*. One and 2 mg. of FU, dissolved in 0.1 *M* sodium phosphate buffer, pH 7.8, was added to homogenates of midguts from untreated females (dissected 24 hours after feeding, *i.e.,* at the peak protease activity). FU did not inhibit protease activity *in vitro*.

The inhibition of protease activity by FU persisted for many days after FU-treatment (Table IV). Protease activity was tested only on the days when all midguts were still filled with blood; the data thus represent the average of the whole population. Though protease activity one day after feeding increases when the blood meal is postponed from two to 7 and 14 days after FU treatment, it does not reach the level of the controls. Inhibition of protease activity was also evident after the second blood meal (76% of the control).

Finally we tested whether blood digestion and protease activity were also inhibited when FU was given in the blood meal. Six-day-old females were fed 0.2% FU through membranes (4 ml. citrated sheep blood were mixed with 1 ml. of 0.85% NaCl containing 10 mg. FU). The results in Table V show that blood digestion was retarded in the treated females, and they also had less protease than the controls. The treated females did not lay any eggs, and their ovaries did not develop beyond stage 4.

Blood digestion in the controls fed diluted citrated sheep blood was slower than in the rat-fed untreated females. The amount of protease one day after membrane feeding was also somewhat lower than after feeding on a rat. Two days after membrane feeding 67% of the controls still had blood-filled midguts, and there was considerable protease activity. All controls laid eggs (an average of 69.5 eggs laid per female) but one day later than after feeding on a rat. The problem whether the delay in blood digestion and maturation of eggs was due to the dilution of the blood by 0.85% NaCl (4:1), the presence of citrate, or the fact that sheep blood rather than rat blood was fed, was not investigated.

Table V

Effect of feeding FU in the blood meal on blood digestion, midgut protease activity, and ovarian development. (FU in sheep blood fed through membranes)

Day after blood meal	% with full midguts		Average proteolytic activity*		Average stage of ovaries**	
	Controls	0.2% FU	Controls	0.2% FU	Controls	0.2% FU
1	100	100	0.61	0.33	5.3	2.7
2	67	92	0.47	0.36	6.8	2.8
3	0	8	—	—	8.0	3.1

* Mg. azoalbumin per three midguts, 12–20 blood-filled midguts in each test.
** Average includes all females, irrespective of the stage of blood digestion.

One week after membrane feeding a second blood meal was given on a rat. Maturation of eggs and blood digestion in the controls were normal; all had stage 8 ovaries and had completed blood digestion 48 hours after feeding. Blood digestion in the treated group was retarded (50% digestion time—61 hours). The recovery of the ability to lay eggs in the treated group seemed to be an all-or-none process. Some females (36%) laid an almost normal number (average 75) of viable eggs, though later than the controls. In the females that did not recover their ability to mature eggs the follicles were small (about the size typical of stage 3–4 ovaries) and of irregular shape.

The relation between protease activity and ovarian development

In normal females the stage of ovarian development at various intervals after feeding on a rat was closely related to the stage of blood digestion (as estimated by the color of the blood mass). When most ovaries have left the resting stage (stage 1) and are in stage 2, blood digestion has just begun; the blood mass is still bright red except for a thin brownish layer around the periphery. By the time the ovaries have reached stage 4–5 only the center retains its red color. In females with stage 6 ovaries the whole blood mass is brown. After that elimination begins.

In FU-treated females blood digestion and ovarian development were retarded to the same degree. Individuals with undigested blood had undeveloped ovaries; in the females where blood digestion had advanced further, the ovaries were also more developed. This holds true for all midguts that were still distended with the blood meal, irrespective of the day of dissection. Females treated with higher concentrations of FU attained a given stage of digestion and ovarian development later than those treated with lower concentrations of FU, or untreated ones.

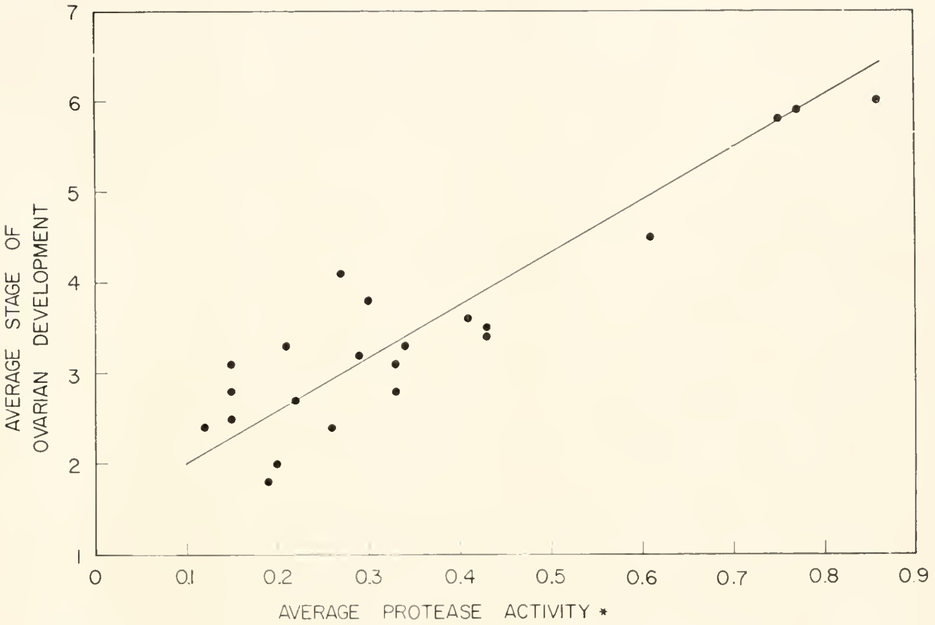

FIGURE 3. Correlation between protease activity and the stage of ovarian development. * Mg. azoalbumin digested by three blood-filled midguts in 30 minutes at 39° C.

The stage of the ovaries of each female whose midgut was taken for the protease assay in Table III was noted (only blood-filled midguts were tested, *i.e.*, before their peak protease activity or near it). In Figure 3 the average stage of the ovaries was plotted against the average protease activity ($n = 22$; 18 experiments from Table III in addition to four experiments with 0%, 0.0125%, 0.025% and 0.05% FU). The correlation coefficient is 0.872; the true correlation at the 95% level of probability is not less than 0.75 (Ezekiel and Fox, 1959). The amount of proteolytic activity is thus closely related to the stage of the ovaries in untreated and treated mosquitoes.

The effect of protease inhibition in vivo on ovarian development

In order to test the effect of protein digestion on the development of the ovaries, we tested the effect of crystalline soy bean trypsin inhibitor (CSBTI) *in vivo.* (Preliminary experiments have shown that CSBTI completely inhibits mosquito protease *in vitro.*) CSBTI was fed to 6-day-old mosquitoes in citrated sheep blood, 625 μg. per ml. Each engorged female thus obtained 1.25 μg. inhibitor in a 2-mg. blood meal.

One day after feeding there was no sign of blood digestion in the treated females; *i.e.,* the color of the blood mass was bright red. Eighty per cent of the ovaries were still in the resting stage (stage 1), only 20% reached stage 2. (The average stage of the control ovaries was 4.4.) Two days after feeding blood digestion began in some of the treated females. The stage of the ovarian development also varied greatly among individual females: 20% were still in the resting stage, the remainder in stages 3–6. The average stage of ovarian development in the treated group was 3.6 as compared with 6.0 in the controls. Three days after feeding the controls had finished blood digestion, and their ovaries contained mature eggs. The delay in blood digestion and ovarian development was still evident in the treated groups three days after feeding. Fifty per cent of the midguts contained blood, 25% of the ovaries were in stage 6, 25% in stage 7, and the remainder contained mature eggs and had empty midguts. Four days after feeding, 7% of the treated females still contained blood. The treated females oviposited later than the controls; the average number of eggs was 37 (48 in the controls). All eggs hatched normally.

The amount of protease in the treated females was 0.17 mg. azoalbumin per three midguts one day after feeding. This probably represents the protease contained in the midgut cells; the protease which reached the lumen was inactivated *in vivo* by CSBTI, since no blood digestion was observed (by the color of the blood mass). Protease activity in the treated group increased on the next day (0.56 mg.). Three days after feeding (when 50% of the treated females still contained blood) the average protease activity in the blood-filled midguts was 0.62 mg.

In untreated females the amount of protease was 0.43 mg. one day after feeding. Two days after feeding all controls contained blood, and the amount of protease was 0.46 mg. (Possibly the two points of protease measurement did not include the peak activity. Ovarian development and blood digestion in the membrane-fed controls in this experiment was more delayed than in the experiment reported in Table V.)

The results of this experiment show that it is possible to inhibit ovarian development by inactivating the midgut protease. It is possible that with higher concentrations of CSBTI, ovarian development could be completely arrested.

DISCUSSION

The results of the present study show that FU inhibits midgut protease production and ovarian development in *Aedes aegypti* females. A similar correlation between the inhibition of ovarian development and midgut protease has been found by Thomsen and Møller (1963) after the removal of the medial neurosecretory cells in *Calliphora*. The trypsin activity in the cockroach, *Nauphoeta cinerea*, is also related to oocyte development (Rao and Fisk, 1965).

The effect of FU in mosquitoes can be interpreted in two ways: (a) The effect of FU on the ovaries might be indirect, due to the deficiency in protease; (b) It is also possible that FU exerts a direct effect on the synthesis of yolk proteins, in addition to its indirect effect.

The results of this study do not enable us to decide between the two possibilities. Our experiments on the feeding of crystalline soy bean trypsin inhibitor have shown that the inactivation of protease *in vivo* suffices to inhibit ovarian development. Other experiments in this laboratory (Weissman-Strum, unpublished results) have shown that the ovaries of *A. aegypti* females fed insufficient amounts of protein develop asynchronously. Such asynchronous ovaries were also observed in FU-treated females. The toxicity of FU in mammals is mainly due to its effect on the alimentary tract (Muggia *et al.*, 1963). If the main damage by FU in mosquitoes is also located in the midgut cells, the production of intestinal protease (and possibly also the absorption of the nutrients) would be impaired. The effect of FU would thus be akin to that of protein starvation.

Studies on the mode of action of FU in microorganisms and experimental neoplasms have shown (see review by Brockman and Anderson, 1963) that in order to become active FU must first be metabolized to fluorouridylic acid and subsequently incorporated into RNA (or be converted to 5-fluorodeoxyuridylic acid which blocks the synthesis of thymidylic acid). It is assumed that FU acts on the rapidly dividing cells in the reproductive system in insects by a similar mechanism (Bǿřkovec, 1962). Our results show that the effect of FU in mosquitoes is not confined to the ovaries.

Incorporation of FU into RNA is known to produce various abnormalities in protein synthesis (Brockman and Anderson, 1963). Incorporation of FU into RNA results in the synthesis of altered enzymes which are enzymatically inactive (Gros and Naono, 1961). Vitellogenesis entails many metabolic processes, involving the synthesis of specific proteins in all parts of the insect body (Telfer, 1965). In *A. aegypti* the yolk proteins are not synthesized in the oocyte itself, but taken up from the haemolymph by micropinocytosis (Roth and Porter, 1964). This uptake is initiated by a blood meal; it is possible that the synthesis of the yolk proteins is similarly triggered. Serological gel-diffusion tests have shown that several proteins, not present in unfed females (and different from the egg proteins), appear after a blood meal in *A. aegypti* (Edman, 1964). These proteins might represent the enzymatic systems which are initiated by a blood meal. Interference by FU with any of these processes which involve the synthesis of specific enzymes, would inhibit the growth of oocytes. Midgut protease might be only one of several enzyme systems which are inhibited by FU, and whose direct result would be the inhibition of egg maturation.

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Summary

1. Oviposition in *Aedes aegypti* females is inhibited by 5-fluorouracil (FU); 0.01% FU fed in the blood meal causes about 99% inhibition of oviposition. The inhibition of oviposition by FU is temporary; some of the females which laid no eggs after their first blood meal recover their ability to oviposit after a second blood meal. The eggs laid by the FU-treated females hatch normally.

2. Oviposition in mosquitoes is also inhibited by feeding FU in sugar solution before the blood meal; 0.05% causes about 95% inhibition of oviposition when given in sugar solution for two days. This effect is also temporary. The eggs laid by females treated with FU in sugar solution hatch normally even when both sexes have been treated.

3. FU slows down the growth of oocytes; the treated females oviposit later than the controls. The rate of oocyte growth and the percentage of females developing mature eggs is proportional to the amount of FU.

4. Blood digestion is retarded by FU-treatment. The delay in blood digestion is already evident one day after feeeding by the color of the blood mass. The period of blood digestion in females treated with 0.05% FU in sugar solution is more than twice that in the controls.

5. Midgut proteolytic activity is inhibited in FU-treated females. The extent of inhibition is proportional to the amount of FU and the time interval between FU-treatment and the blood meal.

6. FU does not inactivate protease *in vitro*.

7. A close correlation between the stage of ovarian development and the amount of midgut protease has been found.

8. Soy bean trypsin inhibitor, which inactivates mosquito protease *in vitro*, also retarded the maturation of eggs *in vivo*.

9. The effect of FU on oocyte maturation in mosquitoes could be an indirect effect (oocyte growth is inhibited because of a lack of midgut protease) or a direct effect of FU on the synthesis of specific proteins in all parts of the insect body.

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STUDIES ON VISCERAL REGENERATION IN SEA-STARS. III. REGENERATION OF THE CARDIAC STOMACH IN ASTERIAS FORBESI (DESOR)[1]

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The ability of sea-stars to regenerate rays following loss or autotomy is too well known to require more than passing mention. Since the replacement structures are complete and normal in all respects, it is obvious that the visceral components of the ray (*i.e.*, the pyloric caeca) can readily be replaced along with the parietal portions. It should not be thought, however, that visceral regeneration is inseparably linked to parietal regeneration; normal pyloric caeca regenerate when only these organs have been operatively removed from an otherwise intact ray (Anderson, 1962, 1965).

Unlike such other sea-stars as *Coscinasterias* and the renowned *Linckia*, *Asterias* does not possess the ability to regenerate extensive portions of the disk (King, 1900). Yet in its feeding activities *Asterias* constantly exposes the largest visceral organ of the disk, the filmy and delicate cardiac stomach, to the hazards of loss or damage by everting it broadly into the external environment. It seems reasonable to expect that the capacity of effecting rapid repair or replacement of this essential feeding organ might have evolved in *Asterias* even in the absence of effective disk regeneration, particularly in view of the ease with which organs are replaced in the rays.

It is my purpose here to present the results of experiments performed to determine whether *Asterias forbesi* is in fact capable of regenerating a cardiac stomach after complete removal of this organ, and to describe the sequence of changes involved in the regenerative process.

It should be borne in mind that the cardiac stomach of *Asterias* is not a simple, baglike organ without significant specializations. The peculiar structural details of this organ were described by Anderson (1954); a brief review of them might be helpful here. Perhaps the most conspicuous features are the elements of the intrinsic retractor system, consisting of a series of muscular and connective-tissue strands ramifying in a consistent pattern over the outer surface of the stomach. These spread from the 5 nodular attachments of the extrinsic retractor harness, branching repeatedly on and eventually in the wall of the stomach, running in the subepithelial connective-tissue layer and disappearing finally near the oral end of the organ. Distributed in a pattern coinciding with that of the intrinsic retractor fibers is a system of branching gutters and intervening ridges; the ultimate branches of the retractors always accompany small gutters. Further related to the gutter-

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ridge pattern is the distribution of cell types in the epithelial lining of the stomach. The gutters are lined by crowded columnar cells with small, ovoid nuclei and single flagella; these will be referred to as "gutter-cells," although in addition to lining the gutters they are found making up the general epithelium in the upper regions of the cardiac stomach. On the shoulder of each ridge between two gutters there is an abrupt transition to a different epithelium, made up of taller, more crowded cells, each almost entirely occupied by a large, dense, elongate nucleus and bearing multiple flagella (2 to 6 per cell). These "ridge-cells" constitute the dominant epithelial cell type below as well as between the gutters, in the region of the "esophagus" becoming intermingled with very abundant secretory cells. This epithelium exhibits a gradual transition to that characteristic of the peristome, which is actually a cuticle-covered epidermis like that clothing the remainder of the body.

It is of interest to determine whether all of these complicated structural details can be duplicated in a regenerating stomach. Further, it is obvious that some of the characteristics—the fibers, the gutters, and the pattern of cell distribution—are closely interrelated. By observing their reappearance in the regenerating organ, it should be possible to draw some conclusions as to whether the interrelated features are also interdependent in development.

The experiments forming the basis of these observations were carried out at the Marine Biological Laboratory, Woods Hole, Mass. It is a pleasure to acknowledge the competent and dependable technical assistance of Helen T. Ghiradella in the conduct of the work.

Materials and Methods

Specimens of *Asterias forbesi* approximately 4 cm. in diameter were obtained from the Supply Department of the Laboratory and kept in running sea water with occasional feeding until needed. For use, a specimen was immersed in $MgCl_2$ solution (8% in tap water) until it was flaccid, when slight external pressure on the rays would cause partial eversion of the cardiac stomach. A fold of tissue just inside the relaxed mouth opening was grasped with forceps while a small incision was made through the peristomial membrane, using iridectomy scissors and working under a dissecting microscope. This incision was then continued around the mouth, freeing the stomach at its oral end and leaving only a narrow margin of peristome bounded peripherally by the nerve ring. The stomach was then gently pulled out, one pouch at a time, and all 5 of the nodular attachments of the extrinsic retractors on the stomach wall were cut. Finally, the cardiac stomach was transected at its upper end, at the level of the constricted opening into the pyloric stomach. Following this operation the specimen was marked for identification with a distinctive application of Nile blue sulfate and returned to running sea water. Recovery was uneventful in most cases, and the animals required no special post-operative care.

For determination of relatively short-term progress in regeneration, specimens were sacrificed at 48, 72 and 96 hours and at approximately one, two and three weeks after removal of the cardiac stomach. In each case the animal was again immobilized by soaking in $MgCl_2$ solution (to prevent autotomy) and its rays cut off, one ray being left slightly longer than the others for purposes of orientation. Fixation was in Bouin's solution, under partial vacuum in a desiccator, and was continued until decalcification of the skeleton was complete. Following standard procedures of dehydration and paraffin embedding, serial sections were prepared at 7–10 μ; the plane of sectioning was arranged so that it passed vertically across the center of the disk in the longitudinal axis of the longest remaining ray-stump. Sections were stained in Mallory's phosphotungstic acid hematoxylin (PTA hematoxylin).

Progress in stomach regeneration beyond three weeks was determined in a different manner, in another series of animals that had been subjected to the same original operation. These specimens, again after treatment with $MgCl_2$ and amputation of the rays, were dissected by cutting around the edge of the disk, transecting the digestive tract at the beginning of the pyloric stomach, and turning back the roof of the disk with the aboral digestive organs attached. This exposed the regenerating cardiac stomach from above and made it possible to examine the extent of regeneration and the degree to which normal relationships with the retractor system had been re-established. For study of the finer details of the regenerated organ the cardiac stomach was freed of all its attachments and either fixed entire or pinned out flat, in segments, in a small wax-bottomed dish, and then flooded with fixative. Fixation in these cases was in Helly's fluid. After paraffin embedding, the organs were sectioned serially at 7 μ in a horizontal plane, thus yielding cross-sections of the stomach. These sections were also routinely stained in phosphotungstic acid hematoxylin.

Return of function in operated animals was checked at irregular intervals after the first week by offering them bits of snail liver or mussel flesh and noting instances of feeding behavior. In some cases animals which were not known to have fed, or which had ignored food when it was offered, were found upon dissection to contain partially-digested prey.

OBSERVATIONS

Removal of the cardiac stomach in the manner described leaves disconnected all the former lines and points of attachment of the stomach: the peristomial membrane, the bottom of the pyloric stomach, and the 5 retractor nodules. In addition, it leaves the perivisceral coelomic cavity in open communication, through the mouth, with the external environment, and the coelomic fluid is lost. Upon recovery from their magnesium-induced paralysis after the operation the animals exhibit a very flat and collapsed appearance; they cling firmly to the substratum, drawing the roof and floor of the disk tightly together, and remain motionless for a day or two. This is undoubtedly a variant of the behavior shown by *Asterias* after autotomy of a ray, when contraction of the body-wall musculature draws together the edges of the resulting wound and makes it possible to seal the opening with a coelomocyte clot.

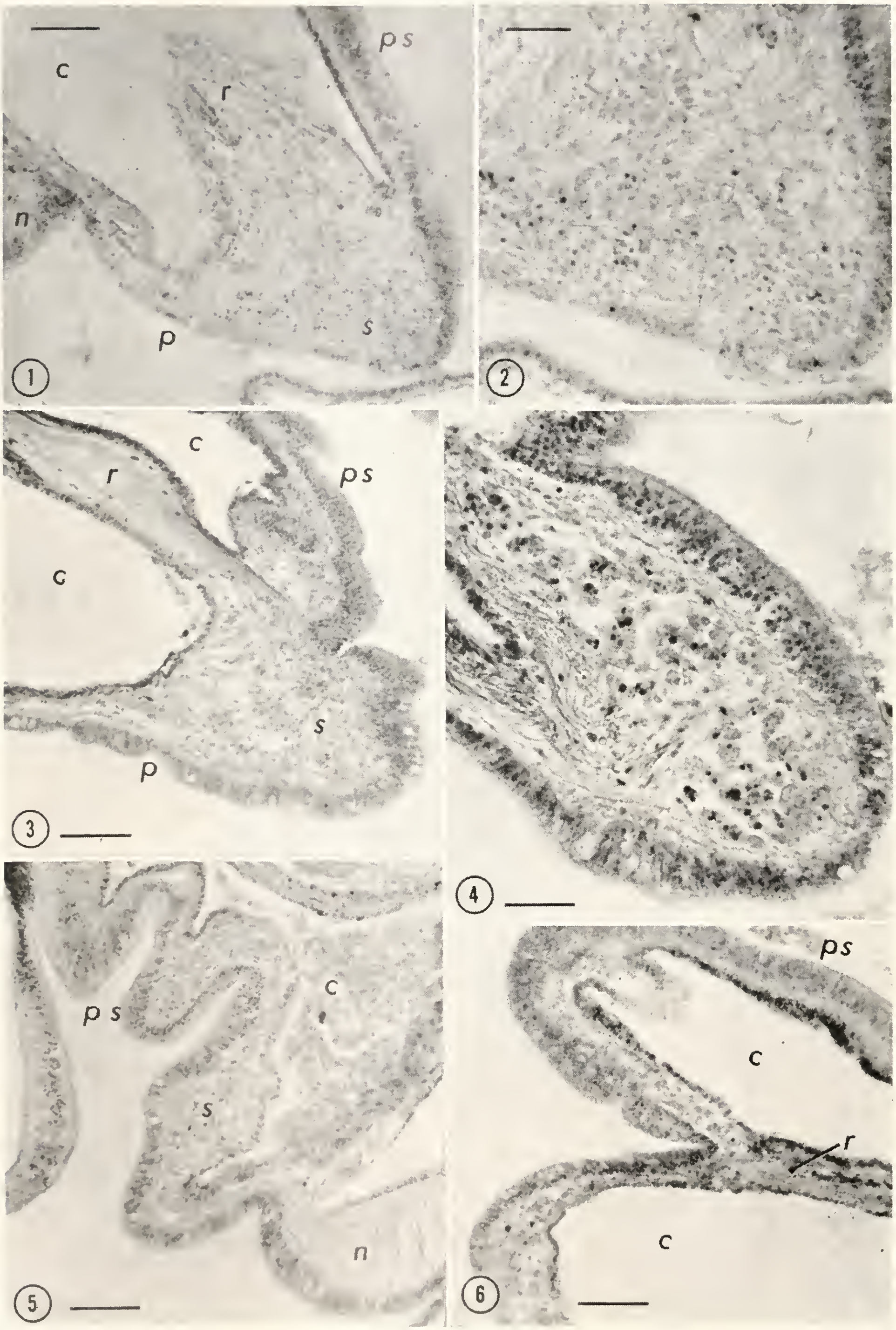
Sections of specimens examined 48 hours after stomach removal show that a similar sealing-off process has by this time restored the integrity of the coelomic cavity. In Figure 1, for example, the edges of the peristome and the floor of the pyloric stomach, as well as a retractor nodule, can be clearly identified, and it is evident that these former attachments of the stomach, previously widely separated, have been drawn together and fused. At this point of wound healing, which we may term the scar area, most of the thick connective-tissue mass has apparently been provided by the retractor nodule incorporated into the scar. In the interradial areas it becomes somewhat thinner. Figures 1 and 2 show also that the continuity of the epithelial lining of the digestive tract has been restored, as a thin epithelial covering has formed over the connective tissue in the scar area. The upper portion of this epithelium, above the sharp bend, is evidently provided by extension of the lining of the floor of the pyloric stomach, while the lower portion apparently owes its origin to the bounding remnants of the peristome. On the coelomic side of the scar area the peritoneum has also been reconstituted as a continuous layer. Between the two epithelia the connective-tissue layer is crowded with cells containing deeply-staining inclusions; these have been interpreted as phagocytic amoebocytes engaged in removal of tissue fragments and debris. Although this point cannot be established with certainty, it seems likely that some of these cells must represent coelomocytes which contributed to the formation of the original wound-sealing clot.

Figure 3 shows a section through a corresponding area in a specimen sacrificed 72 hours after stomach removal. It is evident that the processes of healing and reconstitution noted at 48 hours have simply continued through the following day. The epithelium lining the digestive tract over the scar area is somewhat thicker, better developed, and less disorganized than that seen earlier. As Figure 4 demonstrates, the subepithelial connective-tissue layer in this specimen is somewhat more loosely organized and contains more of the inclusion-bearing cells. There is no indication at 72 hours of any increase in size of the re-forming stomach.

At 96 hours, in contrast (Figs. 5 and 6), the region corresponding to the scar area is considerably more extensive, and the stomach wall has begun a process of folding. By examining the lining epithelium closely it is still possible to determine the point at which the respective contributions of the peristome and the pyloric stomach meet over the thick scar area of connective tissue. Figure 5 shows the appearance of the regenerate in an interradial area, where no retractor is attached; Figure 6 illustrates conditions at the point of junction between a retractor and the stomach wall. It will be noted (and this is generally true in all the 96-hour specimens studied) that the retractor appears to be exerting tension on the stomach, drawing the wall back towards the adjacent ray.

At 96 hours for the first time centers of mitotic activity are in evidence, both in the lining epithelium (Fig. 7) and in the peritoneum (Fig. 8). In both epithelia there are large numbers of relatively undifferentiated cells with large, clear nuclei (*cf.* Fig. 9), and it is apparently these cells that are responsible for the mitotic proliferation leading to the increase in size of the regenerate that is evident by 96 hours. No mitotic activity has been observed in the subepithelial layers, but as seen in Figure 9 there are numerous cells close under the lining epithelium that appear to be laying down horizontally-oriented connective-tissue strands, contributing perhaps to the formation of a new basement membrane.

Figure 10 represents a section through the stomach wall of an 8-day regenerate; the presence of a retractor attachment identifies the region as at least related to the scar area of earlier stages. Several features are noteworthy; for instance, the subepithelial connective-tissue layer is much thinner here than it was seen to be previously under an attachment point, and its fibers run less randomly and in a more oriented fashion. The lining epithelium is very well organized and is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Figures 1–6.

obviously on its way to the restoration of normal appearance; the cells are tall, with basally-crowded nuclei and a conspicuous brush border and flagella. There are still present many cells with large, clear nuclei, however, unlike the situation in a normal epithelium, and mitotic figures are common. At 8 days they are not localized in nests, as they appeared to be at 96 hours (Fig. 7), but are widely scattered, high in the epithelium (Fig. 11). The majority of the mitotic spindles are oriented parallel to the basement membrane, a characteristic which should lead to rapid increase in the surface area covered by the epithelium.

Much lower down in the same 8-day regenerate, in the region that will develop into the peristomial membrane bounding the mouth, differentiation has proceeded in such a way as to produce a normally thick subepithelial layer underlying a typical peristomial epidermis, with its low cuticularized cells. The contrast between this region and the normal stomach wall higher in the organ is well shown in Figure 12, where the two areas are folded so as to lie adjacent to each other. The cell-bodies scattered in the connective-tissue layer of the peristome may represent fibroblasts, but there is a possibility that they are nerve cells, perhaps invading the regenerate from the nearby nerve ring to restore the nerve plexus layer of the gut wall. This 8-day specimen shows the first appearance of specialized neurosensory cells, deeply-staining spindle-shaped bodies lying among the normal epithelial cells; one of these is shown in Figure 12. Such receptors have by this time become fairly numerous in some parts of the stomach lining, forming groups such as those shown in Figure 13.

By the close of the second postoperative week progress towards the replacement of a functional cardiac stomach is striking. Figure 14 shows a section through a small portion of the stomach of a specimen that had been regenerating 15 days, as it lies in contact with a piece of snail tissue over which the stomach had been everted in a completely normal feeding response. Although this stomach is somewhat smaller than normal, the condition of the snail tissue and the presence of what appears to be a food-broth around it indicate that the digestive capacity of the sea-

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FIGURE 1. Overall vertical section of healed zone at one side of the mouth, 48 hours postoperative. Bouin, PTA hematoxylin. Abbreviations: *c*, coelom; *n*, nerve ring; *p*, peristome; *ps*, pyloric stomach; *r*, retractor nodule (passing out of section); *s*, scar area. Note that the continuity of the gut wall has been re-established, and that the peristomial and pyloric-stomach epithelia, clearly distinguishable, have joined to cover the scar area, a thick mass of connective tissue containing many inclusion-laden cells. Scale = 50 μ.

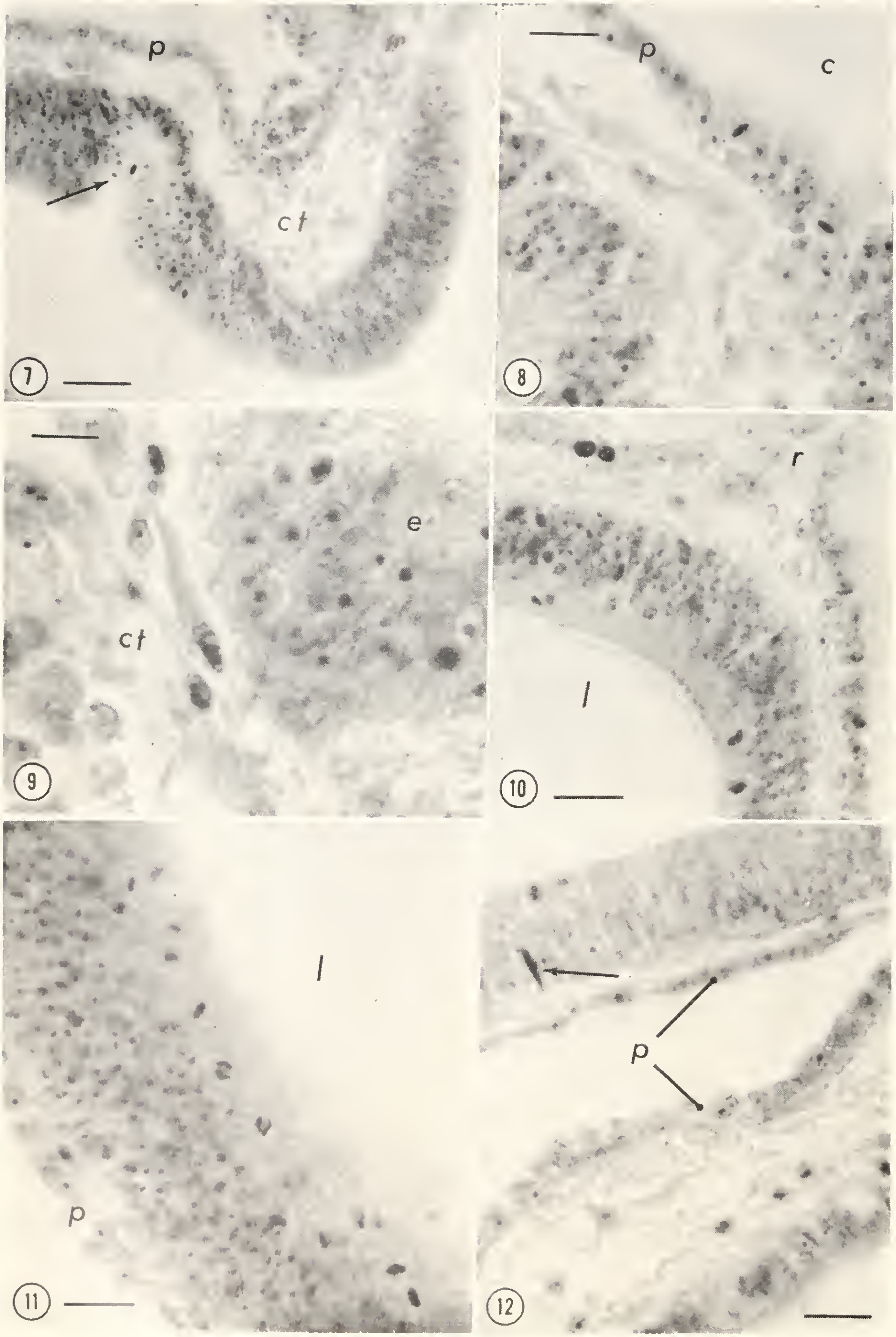
FIGURE 2. Enlargement of the general area marked *s* in Figure 1. Scale = 25 μ.

FIGURE 3. Section comparable to that shown in Figure 1, 72 hours postoperative. Bouin, PTA hematoxylin. Abbreviations and scale as in Figure 1.

FIGURE 4. Scar area from a similar section, 72 hours. Bouin, PTA hematoxylin. Note the persistence of the mass of inclusion-bearing cells in the thick connective-tissue layer. Scale = 25 μ.

FIGURE 5. Section comparable to those in Figures 1 and 3, from opposite side of mouth, 96 hours postoperative. Bouin, PTA hematoxylin. Note the increased extent of the general scar area (*s*) between the pyloric stomach (*ps*) and the nerve ring (*n*). The sagging roof of the pyloric stomach extends down the left side of the figure. The coelom (*c*) contains a mass of coelomocytes. Scale = 50 μ.

FIGURE 6. Section similar to that shown in Figure 5, but passing through the point of attachment of an extrinsic retractor. Bouin, PTA hematoxylin. Abbreviations as before. Note that the retractor appears to exert tension on the wall of the regenerating stomach, drawing it out toward the ray at the right. Scale = 50 μ.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FIGURES 7–12.

star had been restored. Fifteen-day specimens are noteworthy also for the extent to which cell-differentiation has proceeded. Figure 14 illustrates a wrinkled area in the wall of the everted stomach pouch, clothed with crowded, deeply-staining cells (ridge-cells) clearly different from those in neighboring regions; this area also shows localized thickenings of the connective-tissue layer. Figure 15, a section from the esophageal region of a second 15-day specimen, demonstrates again the progress in cell-differentiation characteristic of this stage in regeneration. All of the epithelial components normal for the region are identifiable in this section, and with the sole exception that the subperitoneal muscular layers are not well developed, this tissue exhibits a very normal appearance. The cell types represented in the lining epithelium include ridge-cells, sensory receptors, mucous gland cells, and granular secretory cells.

Figure 16 shows the regenerated stomach of a dissected specimen three weeks after operative removal of the original organ. This specimen had fed normally 12 hours before being dissected, and a mass of partially digested food lies in the stomach. Although no histological details are illustrated, the figure shows the normally wrinkled and folded structure of the organ. Further, the retractor strands have established normal connections with the stomach in all 5 rays (problems of contrast make it impossible to demonstrate this fact in the photograph). Each retractor strand is represented by a stout flat strap, extending from the stomach wall to the apex of the paired triangular extrinsic retractors in its ray.

The stomach shown in Figure 17 was produced by four weeks' regeneration. Although somewhat small for an animal of this size, it is to all appearances a typical cardiac stomach. All of the extrinsic retractors have re-established connections with the stomach wall, and normal outpocketings have begun to be apparent in some of the interradial angles of the disk. When dissected out and spread on wax, segments

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FIGURE 7. Section of a 96-hour regenerating stomach, showing commencement of mitotic activity. Bouin, PTA hematoxylin. Coelom above, lumen of gut below; *ct*, connective-tissue layer; *p*, peritoneum. The arrow indicates a nest of dividing cells in the lining epithelium. Scale = 25 μ.

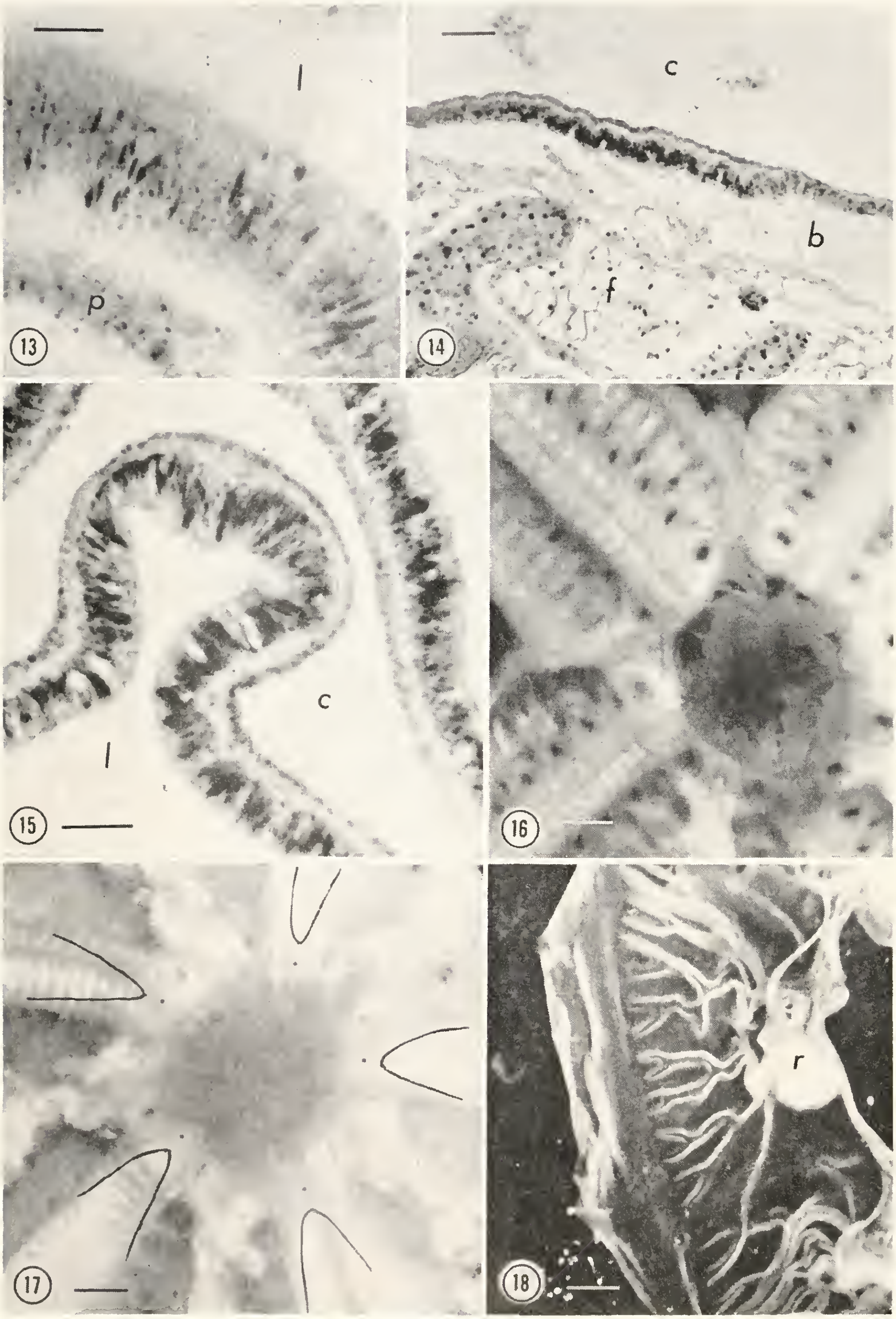
FIGURE 8. Similar section, at 96 hours, showing mitotic activity in peritoneum (*p*) lining the coelom (*c*). Bouin, PTA hematoxylin. Two metaphase figures are clearly distinguishable. The gut epithelium is at lower left. Scale = 10 μ.

FIGURE 9. Section of a small portion of the gut wall at 96 hours, showing cells with unusually large, clear nuclei in the lining epithelium (*e*), underlain by elongated, fibroblast-like cells in the connective-tissue layer (*ct*). Bouin, PTA hematoxylin. Scale = 5 μ.

FIGURE 10. Vertical section of a part of the stomach wall after 8 days' regeneration. Bouin, PTA hematoxylin. The attachment of a retractor (*r*) serves as a reference point. Note that the lining epithelium is much better organized by this time, with basally-crowded nuclei and a normal brush border in evidence, although mitotic activity continues. *l*, lumen of gut. Scale = 25 μ.

FIGURE 11. Oblique section through stomach wall of an 8-day regenerate, showing widespread and intense mitotic activity in the upper part of the lining epithelium. Bouin, PTA hematoxylin; *l*, lumen of gut; *p*, peritoneum. Scale = 10 μ.

FIGURE 12. Section of an 8-day regenerate showing portions of peristome (below) and stomach wall (above) and demonstrating the degree of regional differentiation that has been achieved during the first postoperative week. The thick connective-tissue layer of the peristome is covered externally by a normal cuticularized epidermis and contains numerous cells which may be either fibroblasts or nerve-cells. The peritoneum (*p*) bounds the coelom as a continuous layer. In the stomach lining, the arrow indicates a spindle-shaped body interpreted as a neurosensory cell or sensory receptor. Scale = 10 μ.


13
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p
14
c
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FIGURES 13–18.

of this four-week regenerate revealed details such as those shown in Figure 18. Normal-looking, miniature gutter-and-ridge patterns are evident, each pattern spreading downward from the attachment point of a retractor strand. At this stage in regeneration the gutter-patterns are located quite far aborally in the stomach, and below them there is an unusually extensive stretch of smooth, unadorned wall converging on the peristome (much of this tissue was lost in preparation of the specimen shown in Figure 18). On the whole, the patterned areas occupy a proportionately much smaller part of the stomach than usual. When one of the spread segments is sectioned, approximately at right angles to the long axis of the gutter-pattern, the gutters and ridges are found to show structural details such as those illustrated in Figure 19. The distribution of the two contrasting types of epithelium in the stomach lining corresponds in the normal way to the disposition of the gutters and ridges. In the preparation of this specimen, a portion of the long stretch of stomach wall below the gutters became folded back so as to lie in the plane of the section and thus appears in this figure. It will be noted that the epithelium of this region consists of the type characteristic of the ridges, with its long, densely staining nuclei. The gutters, in a sense, appear to extend elongating fingers of the simpler type of epithelium downward toward the mouth, invading the area of dense cells which then move up and lie on the intervening ridges.

Another four-week specimen, not shown in the figures, regenerated in an instructively anomalous fashion. Here only two of the extrinsic retractor strands reestablished contact with the wall of the re-forming stomach, and only in the sectors of the regenerate pertaining to these retractors has any extensive growth of new stomach occurred. The unattached parts of the organ are smooth and unwrinkled, have not formed pouches, and show no traces of the gutter-patterns which, in con-

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FIGURE 13. Section of gut wall after 8 days' regeneration, illustrating the appearance of groups of spindle-shaped receptors among the cells of the lining epithelium. Bouin, PTA hematoxylin; *l*, lumen of stomach; *p*, peritoneum. Scale = 10 μ.

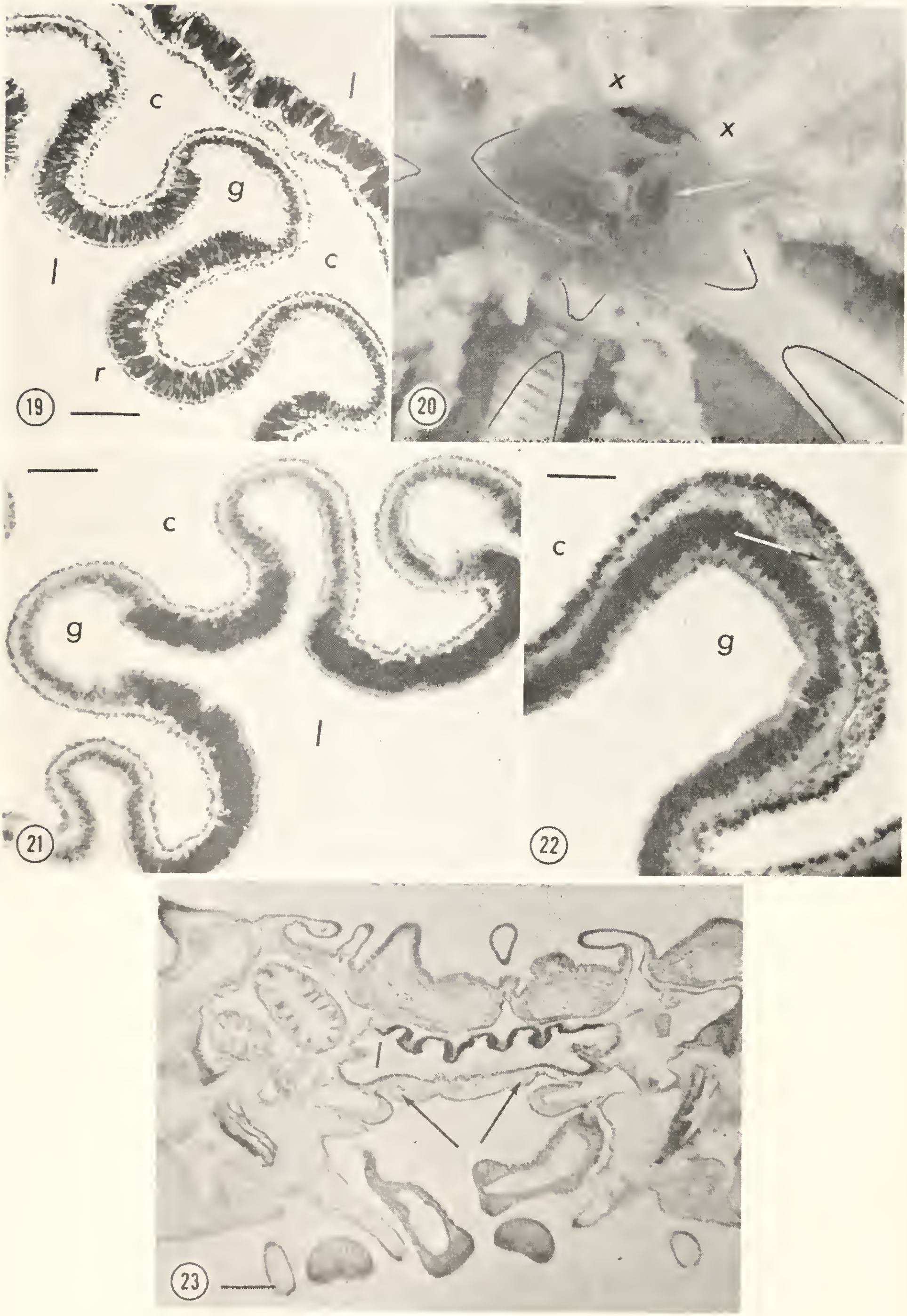
FIGURE 14. Section of a portion of the cardiac stomach of a 15-day regenerate, everted and surrounding partially-digested food (*f*). The stomach wall, passing horizontally across the figure, shows obvious regional differentiations in cell types. It is everted normally in the form of a vesicle filled with coelomic fluid (*c*) and encloses a semiliquid food broth (*b*). Bouin, PTA hematoxylin. Scale = 50 μ.

FIGURE 15. Section of the esophagus in a 15-day regenerate. Bouin, PTA hematoxylin; *c*, coelomic cavity; *l*, lumen of the gut. Two weeks' regeneration has produced an essentially normal epithelium for this region, featuring an abundance of mucous glands and granular secretory cells. Only the subperitoneal muscle net is less well developed than that of a normal specimen. Scale = 25 μ.

FIGURE 16. Aboral view of a dissection, showing the cardiac stomach after three weeks' regeneration. The dark mass inside the stomach is partially digested food; note the folded and wrinkled condition of the stomach walls. Normal retractor attachments have been re-established in all rays, although they are not visible in the photograph. Scale = 3 mm.

FIGURE 17. Another dissection, showing four weeks' progress in replacement of the cardiac stomach. The inked V's outline the outer margins of the paired triangular extrinsic retractors in the 5 rays. All are normally attached to the regenerating stomach, through the regions marked by the inked dots. Scale = 2 mm.

FIGURE 18. A segment of the stomach shown in Figure 17, removed from the specimen and spread on wax, outer (coelomic) surface up. A considerable additional stretch of unpatterned wall, extending toward the left in the picture, was lost in preparing the specimen. Note the normal, dichotomously branching patterns extending orally from the retractor attachment (*r*); though typical in appearance, the gutter-patterns are small and located unusually high in the stomach. Scale = 1 mm.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Figures 19–23.

trast, are well developed in the attached sectors. Sections of the patterned regions of this specimen are entirely comparable, in general structure and in distribution of epithelial cell types, to the section shown in Figure 19. The parts of the stomach wall in which gutter-patterns have not developed are lined by unbroken areas of ridge-type cells.

Figure 20 is an aboral view of a 5-week regenerate which also shows interesting abnormalities. The cardiac stomach was found to contain a tiny snail, dead and decomposing. Healing of the stomach had proceeded in such a way as to leave no passage between the pyloric stomach and the re-forming cardiac portion. As the figure shows, the roof of the cardiac stomach imprisons a gas-bubble, probably produced by decomposition of the snail; although the prey was ingested normally, it could not be reached by digestive enzymes produced in the glandular appendages of the pyloric stomach. Figure 20 also shows that, as in the four-week specimen described earlier, not all of the retractor strands have become reattached to the stomach; in this case two attachments are missing. Close examination reveals further that the bulk of the regenerated stomach is related to the three rays in which retractor attachments have been re-established, very little growth having been produced in the other two rays. As in the previous case, normal gutter-patterns with the expected distribution of epithelial cell types are found only in those portions of the stomach having extrinsic retractor attachments; *i.e.*, there are three sets of gutter-patterns, corresponding to the three retractor attachments. Figure 21 shows a section passing through the region of small, almost terminal gutter branches in one of the patterns. Here there seem to be no differences in the composition of

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FIGURE 19. Cross-section of the stomach-segment shown in Figure 18, approximately at right angles to the long axis of the gutter pattern, at the level of the smaller branches. Helly, PTA hematoxylin; *c*, coelom; *l*, lumen of the gut; *g*, gutter; *r*, ridge. The clear distinction between the type of epithelium lining the gutters (gutter cells) and that covering the intervening ridges (ridge cells) is noteworthy. Gutter cells are restricted to the gutters, at this level in the organ, while ridge cells clothe the smooth wall of the stomach between the gutters and the esophagus; a portion of this epithelium crosses the figure in the upper right corner. Scale = 50 μ.

FIGURE 20. Dissection of an abnormally regenerated 5-week stomach. As in Figure 17, the inked lines emphasize the extrinsic retractors, only three of which have re-established contact with the wall of the stomach. In the two rays marked *x*, no retractors are present, and it can be seen that the part of the stomach on that side is essentially undeveloped. In addition to lacking two retractors, this stomach lacked also an opening into the pyloric stomach. Nevertheless, the animal had ingested a small snail (arrow) which in its decomposition produced the gas bubble trapped in the stomach. Scale = 2 mm.

FIGURE 21. Cross-section of one of the normally-developed sectors of the stomach shown in Figure 20, passing across the small, terminal gutters near the oral end of the gutter-pattern. Helly, PTA hematoxylin; abbreviations as in Figure 19. The structural contrast in the stomach wall as between gutter and ridge regions is striking. At this level, however, no intrinsic retractor fibers run under the gutters. Scale = 50 μ.

FIGURE 22. Similar section from the same specimen, but at a level passing through a major gutter, much higher in the stomach. Helly, PTA hematoxylin; abbreviations as before. At this level intrinsic retractor fibers are clearly to be seen in cross-section just under the peritoneum; the arrow indicates a thick sheet of connective tissue characteristically found at this level between the base of the lining epithelium and the intrinsic retractor fibers. Scale = 25 μ.

FIGURE 23. Vertical section across the disk of an abnormally regenerated specimen one week after stomach removal. Bouin, PTA hematoxylin; *l*, lumen of pyloric stomach. The two arrows indicate the imperforate sheet which has closed the mouth, composed of outer peristomial epidermis, inner epithelium of the floor of the pyloric stomach, with intervening thick connective-tissue layer. This anomaly occurred several times in the course of the experiments. Scale = 2 mm.

the stomach wall as between gutter and ridge, aside from the conspicuous contrast in the epithelia. Somewhat farther aborally, a section passing through one of the major gutters formed by confluence of the smaller branches (Fig. 22) shows that at this level the stomach wall has been invaded by intrinsic retractor bundles. These bundles of fibers lie in their normal relationship beneath the floor of the larger gutters, running longitudinally just under the visceral peritoneum. In the specimen shown in Figure 22, each of the retractors is accompanied by a conspicuous sheet-like thickening of the subepithelial connective-tissue layer of the stomach wall. The intrinsic fibers and the connective-tissue sheets run down under the gutters a considerable distance but eventually dwindle and disappear, before reaching the level represented in Figure 21. As in the previously described specimen, the smooth areas of stomach wall where retractor attachments and gutter-patterns are lacking are lined by a continuous sheet of ridge-type epithelium.

As illustrated by these abnormal 4- and 5-week specimens, failure of one or more of the extrinsic retractors to re-establish connections with the stomach wall is responsible for one of the common anomalies found in regenerated stomachs. A second type of developmental accident, encountered several times in this series of experiments, is shown in Figure 23. This is a vertical section across the disk of a one-week regenerate in which the peristomial epithelium, the floor of the pyloric stomach, and the connective-tissue layer between them have formed an imperforate sheet where the mouth should be. The retractor nodules have apparently not been included in the healing process. In such a situation there is no way in which normal relationships can be re-established, and no further regenerative or restorative progress has been observed in specimens showing this anomaly. One such abnormal specimen survived for about three weeks, without the possibility of feeding; during the third week it successively autotomized all its rays and was finally eaten by other sea-stars in its container.

Discussion

The observations just described provide a clear demonstration that *Asterias* is capable of relatively rapid replacement of an operatively removed cardiac stomach. As early as 15 days after extirpation of the original organ, one specimen was found to display normal feeding behavior, everting a small but apparently complete cardiac stomach over its food and at least commencing the process of digestion. In normal cases, 4 to 5 weeks' regeneration brings the new stomach to such a state of differentiation that it is obviously well on its way to complete development.

It is evident that the "correctness" of the initial healing process is critical for subsequent events in regeneration. If, during the immediate postoperative period of immobility, the edges of the peristome fuse with each other rather than with the cut edges of the pyloric stomach, the mouth is sealed off and no further regeneration is possible. If the junction between peristome and pyloric stomach is properly effected but one or more of the severed retractor nodules fails to be incorporated in the healing area, contact apparently cannot afterward be established, and development of unattached portions of the regenerate is markedly inhibited. We shall return to this point shortly.

The initial sealing-off of the wound, contributing to the adhesion of cut surfaces and restoration of the integrity of the gut wall and the body cavity, presumably involves the formation of a typical asteroid coelomocyte clot according to the process described by Boolootian and Giese (1959). The massive loss of coelomic fluid that occurs during the operation, however, with consequent great reduction in the number of free coelomocytes in the coelom, makes it seem more likely that contributions to the healing of the incision must be made by amoebocytes mobilized from nearby tissues as well as by residual coelomocytes. The intense phagocytic activity in the scar area, lasting through several days after the operation, indicates that large numbers of such amoebocytes must be present. It seems likely also that they and the coelomocytes are involved in fibrogenesis in the connective-tissue layers that are so conspicuous a part of the scar area.

It is interesting that the first indication of growth in the regenerating organ, as distinct from simple immediate wound-closure, is not encountered until sometime during the fourth post-operative day. Mitotic activity, localized at first in nests of cells in the reconstituted lining epithelium, becomes increasingly widespread and intense during subsequent days. The source of the proliferating cells is not apparent; by 96 hours the lining is found to contain many undifferentiated cells with large, clear nuclei, distinct from the epithelial cells which recognizably belong either to the pyloric stomach or to the peristome and have presumably slid over the scar to re-establish the continuity of the lining. The only comparable situation described in the literature of echinoderm gut-regeneration involves the replacement of the entire digestive tract after evisceration in the holothuroid, *Stichopus mollis,* as studied by Dawbin (1949). Here the lumen of a replacement intestine develops by the coalescence of spaces among mesenchyme cells gathered along the edge of the mesentery remaining after loss of the original gut. The lining epithelium of the new digestive tract is produced by proliferation of these cells, but according to Dawbin's account this did not begin until after the 40th day of regeneration; 110 days were required to produce a functional though still immature gut. In *Stichopus,* mitotic activity was widespread in the lining and in the covering peritoneum and appeared to occur throughout the length of the digestive tract. In *Asterias,* likewise, there appear to be no noteworthy concentrations of mitotic activity in the regenerating stomach, although increase in size of the organ is obviously taking place most rapidly in the region of the stomach wall between the peristome and the level of the retractor attachments. The regenerating pyloric caecum of *Asterias* and its close relatives also lacks a definite, localized zone of mitotic proliferation (Anderson, 1965).

Among the most interesting phenomena observed in the experiments on stomach regeneration is the significant morphogenetic role played by the retractor harness. It is apparent that failure of an extrinsic retractor nodule to become incorporated in the healing stomach results in almost complete lack of growth in the related sector of the organ. The effective agent in bringing about normal growth of sectors that have become reattached may be traction exerted by the retractor. Consistently, 96-hour specimens showed that properly attached retractors seemed to be pulling the stomach wall outward toward the rays; such tension would tend to stretch the wall and would probably encourage growth and the addition of new cells.

But failure of growth in the unattached sectors is only part of the story. Where this happens, as we have seen, the branching systems of intrinsic retractors also fail to develop. In the absence of these systems of fibers no gutter-patterns form;

and, most interestingly, where there are no gutters the epithelium completely lacks the types of cells normally associated with them. The causal sequence operating among these obviously interrelated events cannot be established with any confidence on the available evidence. Perhaps it is reasonable to assume, however, that branching patterns of intrinsic fibers radiate from the nodules, spreading over and growing into the wall of the enlarging stomach, in relation to the distribution of tension or traction exerted by the extrinsic retractors. This might have as one of its effects the induction of thicker subepithelial connective-tissue sheets where the pull is transmitted to the stomach wall, and as another, the formation of the associated folds or wrinkle-patterns I have termed gutters. Intrinsic fibers and thick connective-tissue sheets are conspicuously developed underneath the major gutters higher up in the stomach, but the small, terminal gutter-branches may be seen at any moment to extend oralward well beyond these structures. It may thus be argued that perhaps the folds develop as a consequence of tension, and that the intrinsic fibers and connective-tissue thickenings simply follow where the gutter-patterns have already been established. The regularity of the dichotomously branching gutter-patterns is so striking, however, as to make it seem most unlikely that they could have been formed as a simple mechanical folding in response to stress. At this point I can offer no solution to the problem of the relationship between gutter-patterns and fiber-patterns, but the fact that these are closely interdependent characteristics emerges very clearly.

We can proceed to note that wherever even a small, terminal gutter has formed, it is lined by the characteristic gutter-type of epithelium, found otherwise only in aboral regions near the pyloric stomach. Further, the gutter is surrounded by the very different epithelium made up of what I have been calling ridge-type cells. The ridge cells appear to be much more highly specialized than the gutter-cells, yet it is these distinctive cells that are found forming the lining of the relatively undeveloped stomach sectors that lack retractor-attachments and gutters. The relatively miniature, aborally-displaced condition of the immature gutter- and retractor-patterns seen in the four-week regenerates indicates that these systems spread and develop downward, pushing strips of gutter-type epithelium into the previously pure ridge-type lining of the smooth wall. Careful study of sections of the extreme ends of terminal gutters shows the same kind of sudden transition in the epithelium seen at their lateral boundaries: ahead of the gutter, ridge-type epithelium with mucous gland cells; in the gutter itself, nothing but gutter-type epithelium. At 4 and even at 5 weeks, mitotic activity continues in the epithelium at these levels, indicating that cells are still being added and presumably are undergoing differentiation into one or the other type of component, depending on location. There is nothing to suggest that the advance of a gutter brings about transformation of cells lying in its path from one type to the other.

The identity, source, and functional significance of the ridge cells remain to be considered. In an earlier study (Anderson, 1954) the opinion was expressed that these cells, occurring in patches between the gutter-patterns, were probably to be identified with the sensory receptors, cells with cigar-shaped nuclei, described by Smith (1937) in the stomach lining of *Marthasterias*. This line of thought was pursued to a subsequent conclusion (Anderson, 1959) that the absence of patches of cells like these from the cardiac stomach of *Patiria miniata* might indicate that in this species the stomach is relatively insensitive. Still later, however, in studies of the digestive system of *Henricia* (Anderson, 1960), large numbers of spindle-shaped bodies were found that I now think must represent the entities Smith was originally dealing with in *Marthasterias*. These do not, after all, resemble the ridge-cells in the stomach of *Asterias*, but are undoubtedly equivalent to the deeply-staining spindle-shaped bodies that begin to become numerous in the 8-day regenerating stomach (see Figures 12 and 13). The dimensions of these bodies are quite different from those of the ridge-type cells, and they do not bear multiple flagella. In fact, the only point of resemblance between them is the presence in both of a deeply-staining spindle-shaped body. In the receptor this appears to make up the entire cell, while in the ridge-cell it is clearly only a dense, very elongate nucleus. The receptors are widely scattered among other epithelial cells; in contrast, the ridge cells, as we have seen, themselves constitute the entire epithelium in large areas of the stomach wall. This is most evident on the ridges and just below the gutters, and in parts of regenerating stomachs without retractor attachments; the fact that, as pointed out earlier, the ridge cell is actually the basic epithelial cell type in the esophagus as well tends to escape notice because of the very large numbers of secretory cells among which the ridge cells are dispersed (*cf.* Fig. 15). As the gutter cell is in the same sense the fundamental cell type in the more aboral regions of the stomach, the gutter-and-ridge patterns represent areas where the two regionally specialized types of epithelium interdigitate with each other, in characteristic fashion.

The source of the ridge cells in regeneration is problematic. The original population of these cells is completely removed in the operative extirpation of the cardiac stomach. Gutter cells can move down from the edge of the pyloric stomach, in the reconstitution of the stomach lining, but cells that move up from the peristome are not of the ridge-cell type. Yet, while not in evidence on the eighth postoperative day, the ridge-cell patches characteristic of a normal stomach are conspicuously present by the fifteenth day and become increasingly prominent thereafter. The factors inducing or evoking their differentiation cannot be identified. In the portions of the stomach clothed by these cells the relationship of their multiple flagellation to current-production can be understood (although such cells are not found in other regions of the digestive tract or in other systems, where powerful currents are of no less critical importance); the functional significance of their peculiar nuclei, also distinctive, remains without explanation.

SUMMARY

Not later than 48 hours after operative removal of the cardiac stomach in *Asterias forbesi*, the integrity of the gut wall and the coelom is restored by fusion of the cut edges and formation of a thick scar area, over which the epithelia of the pyloric stomach and of the peristome join to produce a continuous lining. Reorganization and phagocytic debris-removal constitute the only apparent changes through the first three postoperative days; by 96 hours, mitotic activity begins, along with increase in size of the regenerating stomach. Growth and differentiation continue rapidly, and as early as the fifteenth day normal feeding and digestion are possible, involving eversion of the regenerated stomach to surround and ingest food. The formation of the normal patterns of intrinsic retractor fibers, and of the gutter-patterns in the stomach wall with which they are associated, requires that

the related extrinsic retractor nodule re-establish proper contact with the scar area at the time of healing. Failure of contact results in marked inhibition of development in the sectors of the stomach involved; no gutter-patterns form, no intrinsic fibers branch over the surface, and the normal interdigitation of distinctive epithelial types characteristic of the gutters and the intervening ridges fails to develop. Study of these developmental anomalies emphasizes the fact that the normal patterns of fibers, gutters, and cell-types are closely interdependent, but evidence to date is insufficient to establish a precise causal sequence of events in their production. It is concluded that the tall cells with multiple flagella and dense, elongate nuclei, localized on the ridges between gutters, are distinct from the spindle-shaped sensory receptors in the stomach lining and in fact represent the basic epithelial cell type in the esophagus and adjacent oral regions of the stomach. Their origin in the regenerating stomach, where they reappear during the second week of regeneration after complete extirpation, as well as the functional significance of their nuclear pecularities, remain obscure.

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THE NATURE OF THE ACTION OF IONS AS INDUCTORS[1]

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Previous work (Barth and Barth, 1959, 1962, 1963, 1964) demonstrated the action of ions as inductors of various types of nerve, pigment cells and neuroglia. The ions Li^+, Mg^{++} and Ca^{++} were particularly effective and Li^+ was shown to induce a sequence of inductions depending upon its concentration and the duration of exposure. This present work deals with experiments designed to test two of the possible modes of action of Li^+ and other ions. The first test is of a destabilizing action of Li^+ and other ions on proteins and DNA. Von Hippel and Wong (1964) have studied the effects of ions on the melting temperature of ribonuclease and have concluded that the effectiveness of various ions is

$$SCN^- > Ca^{++} > Br^- > Li^+ > Na^+ = K^+.$$

Hamaguchi and Geiduschek (1962) found that the thermal denaturing temperature of DNA is lowered by ions in the order of $SCN^- > I^- > Br^-, Cl^-$. Li^+, Na^+ and K^+ are also effective but with minor differences among the three ions.

The second test was stimulated by the report of Ross and Scruggs (1964) that cations decreased the electrophoretic mobility of DNA in the order $Li^+ > Na^+ > K^+$ and $Mn^{++} \gg Mg^{++} > Ca^{++}$. A comparison of the electrophoretic data with the binding of these ions with long chain polyphosphates "supports the idea that the phosphate groups of DNA are the most likely sites of interaction with the alkali metal ions and the particular divalent ions studied" (Ross and Scruggs, 1964, p. 86).

Since the histone-DNA bond appears to be through phosphate, the thought occurs that ions with affinity for phosphate might possibly release histone from DNA and thus activate DNA. The activation of DNA by release of histone is recorded by Huang and Bonner (1962) and the inhibition of DNA activity by added histone is shown by Huang and Bonner (1962) and Allfrey, Littau and Mirsky (1963). Thus, one possible action of ions as inductors would be to activate DNA by competing with histone for phosphate bonds.

METHODS

The methods are the same as described in our previous papers, with four variations. (1) Where relatively high concentrations of an ion (0.1 *M*) were necessary the sodium chloride in our medium was omitted. The sodium chloride in our medium is 0.88 *M*. Therefore, to keep the osmotic pressure at a level consistent with the viability of cells, sodium chloride was omitted in some cases. (2) In order to treat the cells with high concentrations of monovalent cations we used divalent anions such as $SO_4^=$ to keep the osmotic pressure low. (3) In the case of Ca^{++} and

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Mn^{++} we were obliged to omit from our medium the $NaHCO_3$ and the calf serum. Controls treated with our medium lacking $NaHCO_3$ and calf serum differentiated as epithelium. (4) The substitution of lyophilized calf serum (Nutritional Biochemicals Corporation) for globulin from serum (Bios) was made because the latter preparation was no longer available.

A new type of cell is recognized in this study. The cell resembles a unipolar spongioblast and is found in large numbers along with spreading nerve. An entire aggregate may differentiate into these cells. More often there is a mixture of nerve cells and unipolar spongioblasts.

RESULTS

Destabilization of proteins and DNA

Two ions, SCN^- and Br^-, were used to test the possibility that Li^+ was effective as an inductor because of its action as a destabilizer. If so, then SCN^- and Br^- should be more effective than Li^+. LiCl induces a sequence of cell types from nerve to neuroglia at concentrations of from 0.047 *M* to 0.070 *M*. When sodium thiocyanate is added to our medium in concentrations less than 0.05 molar no visible action occurs. If the concentration is increased to 0.074 to 0.084 *M* some spreading nerve is induced but many dead cells result. Thus, possibly the concentration of Na^+ is too high and to test this possibility solutions were prepared with NaCl (0.086 *M*) added. These solutions give the same results as did the addition of 0.084 *M* NaSCN. Therefore the NaCl in the medium was omitted and 0.1 *M* NaSCN added, with the result that the cells survived and differentiated into epithelium and a little nerve. The SCN^- ion is therefore much less effective than Li^+. Similarly NaBr 0.1 *M* if added to our medium produces mostly dead cells, but if substituted for NaCl 0.1 *M* NaBr permits the differentiation of epithelium. In fact the cells do not distinguish between NaCl and NaBr, and cells differentiate normally when cultured in our medium with 0.1 *M* NaBr used in place of 0.088 *M* NaCl. The Br^- ion then is completely inactive at concentrations where Li^+ induces the whole sequence of cell types. The ion series for induction is thus $Li^+ \ll SCN^- > Br^-$, while the series for destabilization is $SCN^- > Br^- > Li^+$.

Comparison of inductive effect and binding of ions to DNA

Ross and Scruggs (1964) studied the effects of ions on the electrophoretic mobility of DNA. For monovalent cations the effectiveness was $Li^+ > Na^+ > K^+$, while for divalent cations it was $Mn^{++} \gg Mg^{++} > Ca^{++}$. At the beginning of the experiments we knew that Li^+ would induce the whole sequence of cellular differentiation from radial nerve to neuroglia. Mg^{++} and Ca^{++} would only induce as far as spreading nerve. Therefore, we tried Mn^{++} to see if it would be more or less effective than Mg^{++} and Ca^{++}.

Table I records the results of experiments in which $MnCl_2 \cdot 4H_2O$ was applied to the presumptive epidermis in varying concentrations and for different intervals of time. Calf serum and $NaHCO_3$ were omitted from the solutions since Mn^{++} precipitates in the presence of these two substances.

In general Mn^{++} has an action similar to that of Li^+ and induces a sequence of cell types depending upon concentration and duration of treatment. In particular

TABLE I

The sequential action of $MnCl_2 \cdot 4H_2O$ *on cellular differentiation*

In this and succeeding tables the headings are to be interpreted as follows.

Stage no.: Shumway (1940); conc.: concentration of substances in milligrams per milliliter of standard solution; hrs.: time in hours during which aggregates are exposed to the substances indicated; types of cellular differentiation: Barth and Barth 1962, 1963, 1964.

Exp.	Stage No.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
1	11−	0.15	2	40	Epithelium
	11−		3	40	Epithelium
	11−		4	40	Epithelium
	11−		cont.	20	Ciliated masses
2	11	0.5	4	50	Mucous cells
			8	50	Mucous cells, nerve cells
			22	25	Mucous, pigment cells
			30	45	Mucous, pigment cells
3	11−	0.5	2	20	Epithelium, radial nerve
			3.5	20	Epithelium, nerve
			5.5	20	Mucous cells, nerve
4	11	1.0	2	20	Epithelium, radial nerve
			3.5	20	Spreading nerve, epithelium
			5.5	20	Pigment cells, spreading nerve
5	11	1.0	6.0	25	Dead cells, pigment cells
			6.6	25	Dead cells, pigment cells
6	11	1.25	0.1	25	Epithelium, radial nerve, spreading nerve
			1.5	25	Spreading nerve, radial nerve
			2.6	25	Spreading nerve
			3.5	25	Spreading nerve
			4.0	25	Pigment cells
			4.5	25	Pigment cells
7	11−	1.25	1.0	35	Radial nerve, epithelium, spreading nerve
			2.1	35	Spreading nerve, epithelium
			4.3	35	Pigment cells, nerve
8	11+	1.25	2.1	25	Spreading nerve, radial nerve, pigment cells
			2.5	25	Spreading nerve, pigment cells
			3.0	25	Pigment cells, nerve
			3.3	50	Pigment cells, nerve
9	11	1.3	0.0	25	Epithelium
			2.1	25	Epithelium, nerve, pigment cells
			2.6	25	Nerve, pigment cells, mucous cells
			3.4	25	Pigment cells, little nerve
			3.6	25	Pigment cells, mucous cells
			4.0	25	Pigment cells, mucous cells

TABLE I (continued)

Exp.	Stage No.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
10	11	1.3	0.0	25	Epithelium
			2.0	25	Epithelium, mucous cells, nerve
			2.5	25	Pigment cells, nerve, mucous cells
			3.0	25	Pigment cells
			5.2	50	Dead cells, pigment cells
11	11	1.5	0.0	35	Epithelium
			1.0	35	Radial nerve
			2.1	35	Spreading nerve, pigment cells
			3.0	35	Dead cells, pigment cells
			3.6	35	Dead cells
			5.0	35	Dead cells
12	11	2.0	0.0	20	Epithelium
			2.0	20	Pigment cells
			2.8	20	Pigment cells
			6.0	20	Dead cells
			6.6	20	Dead cells

a concentration of 1.25 mg./ml. applied from 0.1 hour to 4.5 hours induces first radial nerve, then spreading nerve and finally pigment cells (Exp. 6, Table I). Lower concentrations induce mucous cells as does Li^+. Higher concentrations induce a sequence of cell types but mortality of cells is high. The most effective concentration of $MnCl_2 \cdot 4H_2O$ is about 0.0063 *M*. This compares with a concentration of 0.0016 *M* used by Ross and Scruggs (1964) in their studies on electrophoretic mobility of DNA.

If the phenomena described by Ross and Scruggs (1964) are related to inductive effects, then since Mn^{++} is effective at low concentrations Mg^{++} and Ca^{++} should produce the same effects at higher concentrations if the cells will tolerate them. The addition of $MgSO_2 \cdot 7H_20$ to our medium in high concentrations (0.1 *M*) resulted mostly in dead cells. However when the NaCl was omitted from the medium the cells would tolerate 0.12 *M* Mg and the sequence of inductions previously obtained by Li^+ and Mn^{++} was found (Table II, Exp. 5, 6).

The least effective divalent cation in the study by Ross and Scruggs (1964) was Ca^{++}. Although previous investigations (Barth and Barth, 1962) showed that Ca^{++} would induce radial and spreading nerve, no pigment cells were obtained. We, therefore, tested higher concentrations of Ca^{++} and encountered the difficulty that Ca^{++} precipitated in our medium. By omitting calf serum and $NaHCO_3$ we were able to use higher concentrations of Ca^{++} than previously used. In order to keep the osmotic pressure within the limits of viability of cells we also omitted NaCl. The results of the application of various concentrations of $CaCl_2 \cdot 2H_2O$ for varying lengths of time are recorded in Table III. The data show induction of spreading nerve, slate grey epithelium and pigment cells by Ca^{++} at concentrations of 12.0 mg./ml. and 15.0 mg./ml. Thus, Ca^{++} is effective as an inductor at concentrations of from 0.081 to 0.1 *M*. These concentrations are somewhat lower

TABLE II

The sequential action of $MgSO_4 \cdot 7H_2O$

Exp.	Stage No.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
1	11	18.0	1.2	20	Nerve, epithelium
			2.0	20	Spreading nerve
			4.0	20	Dead cells, nerve
2	11	20.0*	2.0	35	Spreading nerve
			3.0	40	Spreading nerve, dead cells
			4.0	35	Dead cells, nerve
3	11	25.0	1.6	35	Spreading nerve, other nerve
			3.0	40	Spreading nerve, UPS
			3.5	35	Spreading nerve, UPS
			4.0	40	Spreading nerve, UPS
4	11	30.0	1.8	25	Spreading nerve
			2.3	25	Spreading nerve
			2.8	25	Spreading nerve, UPS
			3.3	25	Spreading nerve, UPS
			4.0	25	Spreading nerve, UPS
			6.0	25	Spreading nerve, UPS
5	11	30.0	3.1	25	Spreading nerve
			5.0	25	Spreading nerve
			7.7	25	Slate-grey epithelium, nerve, pigment cells
			8.5	35	Slate-grey epithelium, pigment cells, nerve
6	11	30.0	8.3	25	Nerve, slate-grey epithelium
			9.0	50	Spreading nerve, slate-grey epithelium, pigment cells
	11	35.0	3.5	25	Spreading nerve, UPS
			8.3	25	Slate-grey epithelium, nerve
			9.0	25	Slate-grey epithelium, nerve, pigment cells
7	11+	35.0	2.1	25	Spreading nerve, slate-grey epithelium
			5.4	25	Slate-grey epithelium, nerve
			7.8	25	Nerve, pigment cells
			8.0	45	Pigment cells, nerve

* $MgCl_2 \cdot 6H_2O$ used in this experiment.
UPS = unipolar spongioblasts.

than the effective concentrations of Mg^{++} and the series for induction becomes $Mn^{++} \gg Ca^{++} > Mg^{++}$.

In assessing the value of the comparison it must be pointed out that in our studies, Mg^{++} was used in the presence of calf serum and $NaHCO_3$, while Ca^{++} was applied in the absence of these substances. Undoubtedly there was some binding

TABLE III

The sequential action of $CaCl_2 \cdot 2H_2O$

Exp. no.	Stage no.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
1	11−	10.0	2.1	15	Nerve, epithelium
			4.4	15	Nerve
2	11	10.0	1.0	25	Spreading nerve, epithelium
			3.0	25	Nerve, slate-grey epithelium, UPS
			3.8	25	Spreading nerve, slate-grey epithelium, UPS
			8.8	75	Slate-grey epithelium, pigment cells, spreading nerve
3	11	12.0	8.8	40	Nerve, dead cells
			9.8	42	Dead cells
4	11+	12.0	4.8	35	Slate-grey epithelium, UPS, nerve
			7.0	45	Slate-grey epithelium, pigment cells, nerve
5	11	12.0	3.5	35	Spreading nerve, UPS, pigment cells
			6.3	40	Pigment cells, nerve, slate-grey epithelium
			6.6	35	Pigment cells, nerve
			20.0	40	Dead cells
6	11	12.0	5.0	40	Slate-grey epithelium, pigment cells, UPS, nerve
			7.2	42	Pigment cells, slate-grey epithelium, nerve
			7.8	48	Pigment cells, slate-grey epithelium, nerve
			8.4	40	Pigment cells, nerve, dead cells
7	11+	12.0	7.3	25	Spreading nerve, slate-grey epithelium, pigment cells
			22.0	25	Dead cells
8	11	15.0	2.7	25	UPS, spreading nerve
			4.5	25	UPS, spreading nerve
			7.5	25	UPS, spreading nerve
			8.2	75	Spreading nerve, UPS, dead cells
9	11	15.0	3.3	35	UPS, spreading nerve
			4.3	40	UPS, spreading nerve
			4.8	35	UPS, spreading nerve, dead cells
			5.3	40	UPS, spreading nerve, dead celss
10	11	15.0	4.8	35	UPS, spreading nerve
			5.3	40	UPS, spreading nerve
			7.0	35	UPS, spreading nerve
			8.0	40	UPS, spreading nerve

* Not typical.
UPS = unipolar spongioblasts.

TABLE III (continued)

Exp. no.	Stage no.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
11	11+	15.0	7.3	25	Nerve, slate-grey epithelium, pigment cells
			22	25	Dead cells
12	11+	18.0	3.0	40	UPS, spreading nerve
			4.0	42	UPS, spreading nerve
			4.5	40	Spreading nerve, UPS
			5.3	42	Spreading nerve, UPS
			7.4	20	Spreading nerve, UPS, dead cells
13	11	18.0	2.8	25	Radial nerve
			4.2	25	Spreading nerve, Ca^{++}-induced nerve
			5.0	25	*Spreading nerve, UPS
			8.8	25	*Spreading nerve
14	11+	20.0	4.7	40	Spreading nerve, UPS
			7.0	36	Dead cells, spreading nerve
15	11	20.0	2.8	25	Radial nerve
			4.2	25	Spreading nerve, Ca^{++}-induced nerve
			4.0	25	*Spreading nerve, UPS
16	11+	25.0	2.0	25	Radial nerve, spreading nerve
			3.0	25	Spreading nerve, UPS
			3.2	25	Spreading nerve, UPS
			3.5	30	UPS, Spreading nerve
			3.8	30	Dead cells, UPS, nerve
			4.2	40	Dead cells

of Mg^{++} by calf serum at pH 8.0 while there was no such binding possible in the case of Ca^{++} because the calf serum was omitted. This difference in the use of Mg^{++} and Ca^{++} is a fault in the design of the experiments whereby Mg^{++} was used first and when it was found that Ca^{++} would not remain in solution with calf serum and $NaHCO_3$, the latter substances had to be omitted.

With the above comment in mind, there is a close enough resemblance between our studies on induction and those of Ross and Scruggs (1964) to warrant further study. We, therefore, examined the effects of monovalent cations which Ross and Scruggs rate as $Li^+ > Na^+ > K^+$ in relative binding strengths of 1.5 to 1.0 to 0.8. The concentrations used were of the order of 0.02 M for Li^+. We find that Li^+ at this concentration induces a sequence of cell types after the cells are exposed for long periods (24–48 hours). Actually it is more expedient to use higher concentrations of Li^+ (0.1 M) for shorter periods of time. Therefore, we would need to apply concentrations of from 0.15 M to 0.2 M Na^+ or K^+ to obtain inductions similar to those of Li^+. Such concentrations attained by either NaCl or KCl are too high for the viability of cells, possibly because of the high osmotic pressure.

TABLE IV

The sequential action of K_2SO_4, Na_2SO_4 and LiCl

Exp. no.	Stage no.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
1	11	8.7K$^+$	4.0	40	Epithelium
			18.0	30	Dead cells, mucous cells
2	11+	8.75K$^+$	4.0	35	Epithelium
			6.0	35	Epithelium, mucous cells
3	11	17.5K$^+$	3.0	25	Radial nerve
			4.0	25	Spreading nerve
			8.0	25	Dead cells, spreading nerve
			8.5	75	Dead cells, rare nerve
4	11+	14.2Na$^+$	4.8	35	Spreading nerve
			5.8	40	Spreading nerve
			6.5	35	Dead cells, spreading nerve
5	11	14.2Na$^+$	4.0	35	Radial nerve, spreading nerve, epithelium
			5.0	40	Spreading nerve, radial nerve
			6.0	35	Spreading nerve
			22.0	35	Dead cells
6	11	20.0Na$^+$	3.0	25	Radial nerve, spreading nerve epithelium
			7.8	25	Dead cells
7	11+	20.0Na$^+$	5.0	25	Nerve, dead cells
			5.7	25	Dead cells, nerve
			6.2	25	Dead cells, nerve
			9.1	25	Dead cells
8	11	4.0Li$^+$	1.3	25	Radial nerve, spreading nerve, epithelium
			2.0	25	Spreading nerve, epithelium, rare pigment cells
9	11	6.0Li$^+$	1.0	25	Spreading nerve, radial nerve, slate-grey epithelium
			2.0	25	Pigment cells, spreading nerve
			4.3	25	Pigment cells
10	11	8.0Li$^+$	1.0	25	Slate-grey epithelium, spreading nerve
			2.0	25	Pigment cells
			4.3	25	Dead cells, pigment cells

In any case we found that the cells would tolerate up to 0.28 M Na$^+$ if applied as Na_2SO_4 and up to 0.2 M K$^+$ if applied as K_2SO_4.

Table IV compares the effects of K$^+$, Na$^+$ and Li$^+$ added to our medium in which NaCl is omitted. First, it can be seen that K$^+$ and Na$^+$ do not induce the entire sequence of cell types as does Li^+. K^+ and Na^+ will induce mucous cells, radial nerve and spreading nerve, but not pigment cells. The effectiveness of K^+, Na^+ and Li^+ as regards the induction of spreading nerve is $Li^+ > K^+ = Na^+$. Ross and Scruggs (1964) report Na^+ as slightly more effective than K^+. As regards pigment cell induction, either it is not possible to expose the cells to a concentration of K^+ or Na^+ high enough to induce pigment cells, or the mechanism of induction of pigment cells is not related to the binding power of these ions.

So far as the monovalent cations are concerned, there is evidence of additive effects of any two cations. For example if the complete medium is used containing 0.088 *M* NaCl, then less Li^+ is required for the induction of pigment cells (0.074 *M* as compared with 0.10 *M* when NaCl is absent). Similarly 0.1 *M* K^+ has no effect by itself but if applied in the presence of 0.088 *M* NaCl, induction of radial nerve, spreading nerve and unipolar spongioblasts is obtained.

Discussion

The correlation between the destabilizing effect of various ions on proteins and DNA does not support the hypothesis that ion inductors function by means of a destabilizing effect. However, this finding cannot be construed as evidence against the hypothesis, for there is the question of whether the ions penetrate the cell and reach the DNA. The fact that NaBr will substitute for NaCl in our culture medium may possibly indicate that the Br^- ion does not penetrate.

In addition, the concentrations used for destabilization of DNA were of the order of 1.0–4.0 *M*, whereas the concentrations used to test inductive capacity were of the order of 0.1 to 0.2 molar. We conclude, therefore, that while we have no evidence for the hypothesis we cannot exclude it.

The correlation between the combining power of various ions with DNA and their inductive capacity is a very close one. There is good correspondence of the order of effect of monovalent and divalent cations as regards their inductive effect and their binding with long chain polyphosphates. In addition, the order of magnitude of the concentrations used in both sets of experiments is the same. There still remains the question of the effective intracellular concentration of the ions in the experiments on induction. We do not know the actual concentrations of the ions in the cell nor the distribution of the ions within the cell. Experiments are planned to yield this information.

Granting that the ions penetrate, the combining sites are not known. Long chain polyphosphates other than DNA may combine with the ions. In addition the ions may possibly combine at sites other than the phosphates of DNA and although the amount bound would be much less, nevertheless this type of binding may be the effective one in activating DNA.

The general hypothesis would state that ions act as inductors by competing with histone for phosphate sites on DNA. The resulting dissociation of histone from DNA would progressively activate DNAs.

In evaluating the extent to which the hypothesis applies, we must consider the problem of the action of proteins as inductors. How could they produce an effect by altering phosphate binding? At first sight there seems to be very little in common between the actions of ions and proteins. However, two possible modes of action of the proteins could lead to binding of DNA phosphate. If the proteins used as inductors penetrate the cell as protein molecules, these would compete with histone for the phosphate sites on DNA or, if acid, proteins would compete with the DNA phosphate for alkaline sites on the histone. In either case dissociation of the DNA-phosphate-histone would be increased.

Vainio, Saxén, Toivonen and Rapola (1962) found that bone marrow proteins penetrated ectoderm cells as tested by their combination with fluorescent antisera. They find no definite concentration of the fluorescent antigen in the nuclei but rather a uniform distribution. Yamada (1962), reporting experiments by Okada and K. Takata, states that fluorescent antisera show an intense staining of the cytoplasm, especially on the surface of yolk granules. Some staining of the nucleus was also observed.

Another possible explanation of the action of proteins as inductors stems from the fact that they are applied to the ectoderm as precipitates. The proteins are soluble in weak salt solutions and would be expected to go into solution. Thus, a saturated solution of protein would be in contact with the cell membranes. It follows that water must be withdrawn from the cells and this would lead to an intracellular concentration of the Na^+, K^+, Mg^{++} and Ca^{++} ions present. This increase in concentration of ions may possibly be enough to induce nervous tissues by the mechanism of phosphate binding.

In this connection we wish to report some experiments with a 0.17 *M* sucrose solution which induces a sequence of cell types beginning with radial nerve and ending with pigment cells. The effects of 0.17 *M* sucrose are the same as those of Li^+, Mn^{++}, Mg^{++} and Ca^{++}. A tentative interpretation of the effect of sucrose is offered. Sucrose withdraws water from the cell and as a result the internal concentration of the ions is increased to a point where induction occurs. More work with high molecular weight compounds is necessary to test this idea.

SUMMARY

1. A comparison of the effects of ions as inductors, on the one hand, and on destabilization of proteins and DNA, on the other, shows a lack of correspondence as regards the effectiveness of various ions.

2. A comparison of the effects of various monovalent and divalent cations as inductors with their effects on the electrophoretic mobility of DNA reveals a close correspondence as regards the effectiveness of the ions.

3. An hypothesis of the nature of the effect of cations as inductors is presented. Cations act to combine with the phosphate groups of DNA, thereby removing it from the inhibiting action of histones.

4. A possible interpretation of the action of proteins as inductors by increasing the intracellular concentration of cations is offered.

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EFFECT OF SEX HORMONES ON GONADAL DIFFERENTIATION IN A CICHLID, TILAPIA AUREA

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In recent years *Tilapia* has attained great economic importance, as it might be an excellent fish for cultivation in ponds. It feeds directly on plankton, grows rapidly, multiplies abundantly and flourishes in brackish water. It tolerates salinities as high as 60% sea water without adaptation, and 160% sea water after a short period of adaptation (Lotan, 1960). However, the remarkable fecundity of *Tilapia* has proved to be a serious drawback. The pond-raised fish become mature and start to breed when they are still very small—40 gm. and even less. As a result, the water of the pond swiftly becomes crowded with fish too small for eating (for review, see Hickling, 1963).

The purpose of the present investigation was to prevent reproductive processes in *Tilapia,* by sterilization, in order to stock fish ponds with sterile fry. This was done by means of sex hormone treatment during the stage of gonadal differentiation.

Sex differentiation in teleosteans has been observed mainly in cyprinodonts and in a few other groups (Wolf, 1931; D'Ancona, 1943, 1957; Ashby, 1957, 1959; and others). The cichlids have hitherto not been examined in this respect. Therefore, it was apparent that a knowledge of gonadal differentiation of *Tilapia* was necessary for the sterilization experiments.

The part of the study reported here is restricted to the normal differentiation of the gonads of *Tilapia aurea* and the effect of sex hormones on this differentiation.

Material and Methods

The fish, *Tilapia aurea,* serving for these observations were obtained and bred at the Fisheries Research Station, Dor. Mature males carrying fertilized eggs in their mouths were captured in the ponds of the Station and kept in aquaria. The water in the aquaria was changed daily. The day when the brood was released from the mouth at the end of the incubation period is referred to as the day of hatching. Samples were withdrawn for histological observations from the day of hatching, for two months, during weekly intervals.

For the hormonal treatment, mature fish carrying fertilized eggs in the oral cavity were put into concrete pools with the dimensions of 2 × 4 × 0.7 meters. At the age of 4–5 weeks, the small fish were introduced into 30 liters of fresh water in fiberglass tanks of about 45 liters capacity—100 fish in each tank. All the hormones, except the stilbestrol-diphosphate which is water-soluble, were dissolved in ethanol at a concentration of 1.0 mg. per ml., and slowly poured into the tank. The contents of the tanks were renewed every day. The fish were kept in the hormone solutions for 5–6 weeks.

The concentrations of hormones used were: 50, 100, 200, 400, 800 and 1000 μg.

per liter water. These high concentrations were chosen because in previous experiments doses lower than 50 μg. per liter, from hatching, for a period of three weeks, had no effect whatsoever on the gonads of *Tilapia*. The controls, treated with the same concentrations of ethanol used to solubilize the hormones, showed no abnormalities and are included within the group of the untreated animals. All cultures were fed on yeast powder and corn flour.

At weekly intervals, samples of five individual fish were removed from the tanks and fixed in Bouin's fluid. An incision was made in the abdomen in order to facilitate the penetration of the fixative. The gonadal area was serially sectioned at 8 μ thickness and stained with Ehrlich's haematoxylin and eosin.

The total length, measuring from the snout to the end of the caudal fin, is given for size of fish.

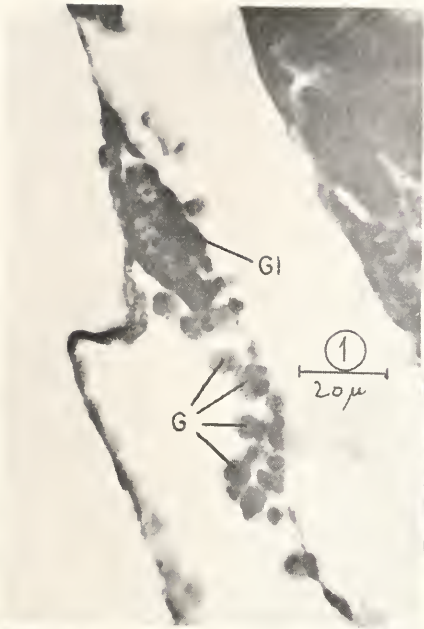
The hormones used were: stilbestrol, stilbestrol-diphosphate-diaethyldioxystilben-diphosphate (Honvan, ST-52-Asta), testosterone and methyl testosterone. All these compounds were commercial preparations.

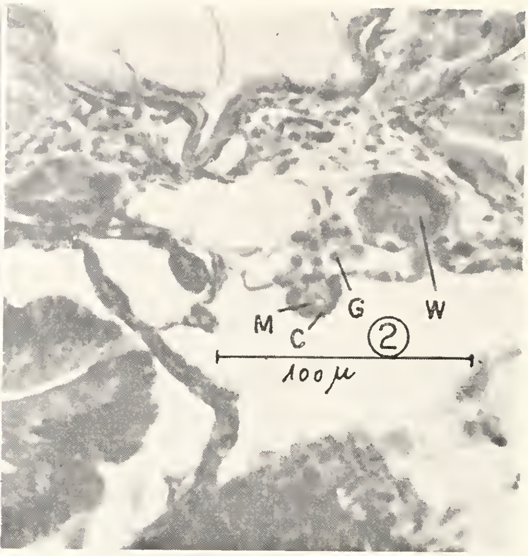
Results and Discussion

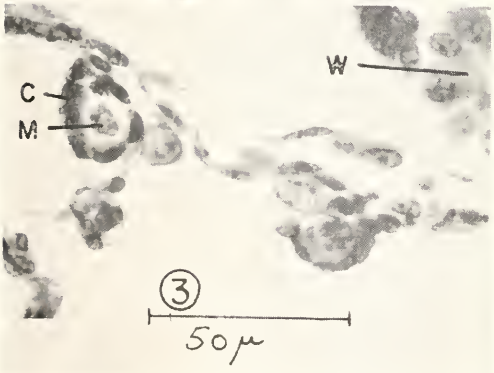
Gonadal differentiation in untreated animals

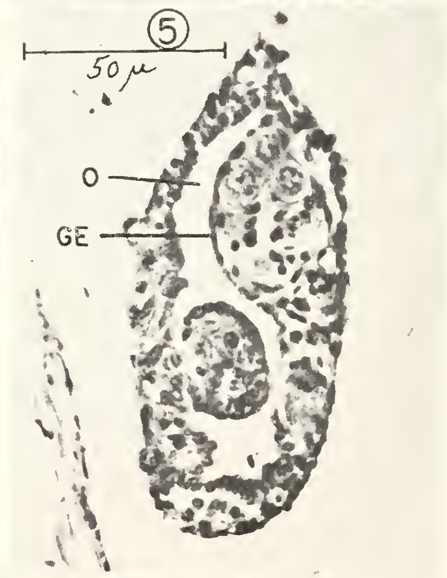
In the first stage examined, the fish, 2–3 days after hatching, had a length of 5–6 mm. A large yolk sac was attached to the abdomen. At this stage, the genital ridge could not be found. Only 8 days later a definite genital ridge was seen. It is known that much, or even all, of the development of the gonads in teleosteans occurs after hatching. Therefore, the first appearance of the gonad at 10–11 days after hatching in *Tilapia* is a remarkable delay. Only in *Gobius* is this record surpassed; there the gonads appear at 15 days of age (Forbes, 1961). The genital ridge consisted of two epithelial layers, formed by the folding of the peritoneal lining, emerging on stalks from the somatopleura into the body cavity. In cross-sections through the gonadal region, large quantities of undigested yolk were found within the abdominal cavity. The genital ridge spreads from the middle of the air bladder to near the urogenital papilla. The two contralateral parts of the genital ridge are separated anteriorly and suspended on two separate ligaments from the somatopleura. More caudally, they become gradually attached to the mesenterium.

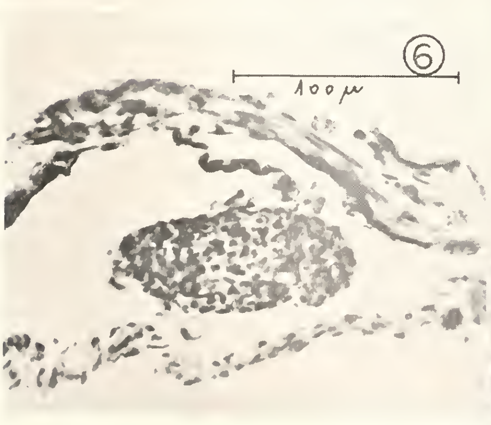
The fish reached a length of 7.5–8.5 mm. at the age of 16 days. Gonocytes (germ cells) could be seen on the splanchnopleura, and at the junction of the somatopleura with the splanchnopleura. The gonocytes approached the gonadal anlagen from the lateral somatopleura only at about the caudal quarter of the gonad. Here, clusters of gonocytes were seen near the Wolffian ducts (Figs. 1, 2). Accordingly, the path of migration of the germ cells in *Tilapia* differs from that found by Wolf (1931) and confirmed by Tawolga (1949) in *Platypoecilus caudatus*. In the latter, the main path of migration is the lateral part of the somatopleura. The gonocytes can be easily distinguished from all other cells by (1) their large size, (2) the indistinctiveness of their nuclei, and (3) the presence in most of them of numerous yolk spherules (Figs. 1, 2). The gonadal anlagen now contain three kinds of cells: large distinctive gonocytes, the peritoneal cells bounding the anlage and stromal cells scattered in among the gonocytes. The nuclei

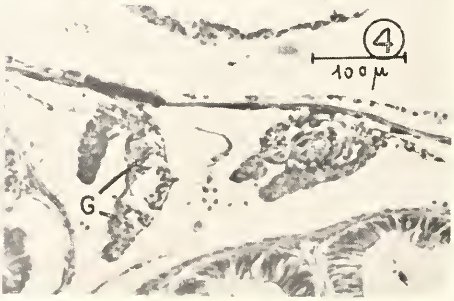












Figures 1–6.

alone, of these two somatic constituents of the gland, are visible. More caudally, the gonadal anlage consists of two separate layers, *i.e.*, cortex and medulla (Figs. 2, 3). From the present observations it is not clear whether this medulla represents a medulla in the sense adopted for other classes of vertebrates, namely, whether it originates from the underlying mesenchyme and from the mesonephric rudiments (Witschi, 1950). According to D'Ancona (1957) among the teleosteans, only the hermaphroditic teleosteans have a medullary component in their gonadal anlagen.

In fish 30 days of age, 10–12 mm. in length, the gonadal anlagen are well developed. Blood vessels are beginning to make their appearance. At this stage the first discrimination between the sexes can be made, according to a longitudinal groove that appears only in presumptive ovaries. With these grooves the ovaries appeared as two parallel ridges of different height which emerge from a joint stalk (Fig. 4). Only the median ridge contained the gonocytes, which were located between the two peritoneal linings. These two ridges fuse at their ventral peaks and enclose between them a cavity lined with peritoneal covering, the germinal epithelium, which forms the ovocoel (Figs. 4, 5).

At 39 days the fish were 12.5–16 mm. long. The gonads were at some places constricted in the dorso-ventral plane. The gonocytes were the predominant element. While they were spread throughout the body of the gland, the stromal cells were restricted mainly to the outer part of the gland.

At 7–8 weeks of age, with the body length of 18–22 mm., gonadal sex differentiation took place. The ovary differentiates a week to 10 days before the testis. The most distinctive female characteristic is the enlargement of the germ cells to two to three times their former size. The stromal cells become pressed to the periphery. Therefore, the center of the gland is occupied mostly with stromal cells. The identification of the gonocytes as such becomes impossible soon thereafter (Fig. 6). This description was reported also for *Xiphophorus* (Essenberg, 1923) and for *Platypoecilus* (Wolf, 1931). After the first definite stages of testicular development, there was complete quiescence which continued unbroken even in the largest and oldest male studied. A similar situation, namely, testicular quiescence after the first stages of differentiation, was observed in *Salmo trutta* by Ashby (1959).

Modifications induced in gonads by hormone treatment

The hormone treatment was begun when the fish were 4 to 5 weeks old. As sexual dimorphism takes place at the age of 7–8 weeks, the treatment preceded the stage of gonadal differentiation.

All figures are of transverse sections with animal's right on right-hand. The scale indicating magnification is shown on each figure. Figures 1 to 7 are untreated controls.

FIGURE 1. Gonocytes (G) on the splanchnopleura near the germ gland anlage (Gl), at 16 days.

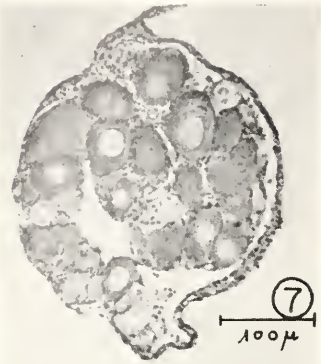
FIGURE 2. Gonocytes (G) between the Wolffian duct (W) and the caudal quarter of the germ gland anlage (Gl), at 16 days.

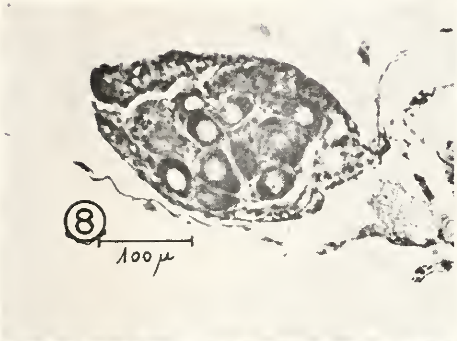
FIGURE 3. Caudal part of the germ gland anlage (Gl) at 16 days, showing distinction between cortex (C) and "medulla" (M).

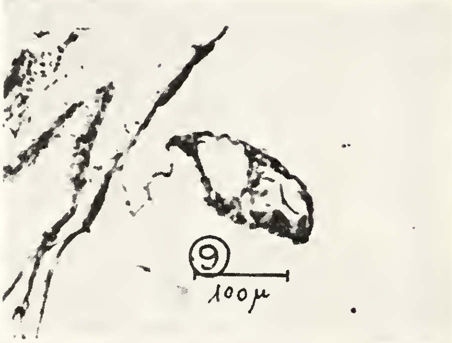
FIGURE 4. Ovary at 30 days, composed of two ridges of different height. Only the median ridges contain gonocytes (G).

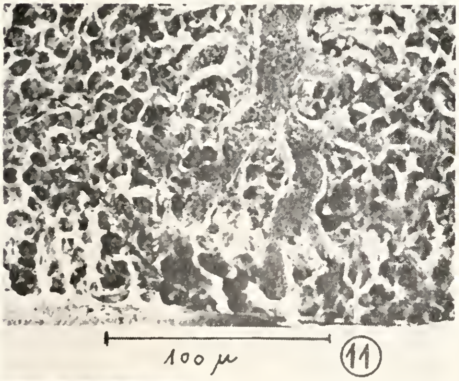
FIGURE 5. Ovary at 36 days, showing ovocoel (O) lined with germinal epithelium (GE).

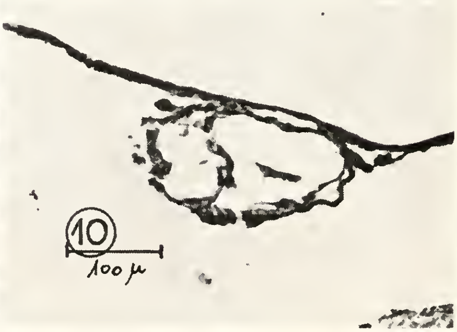
FIGURE 6. Testis at 10 weeks.

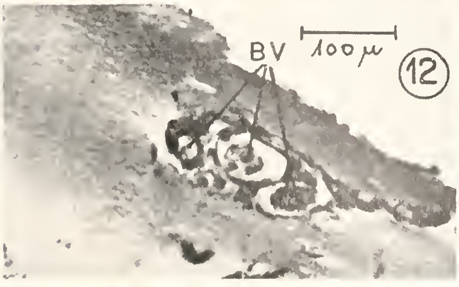


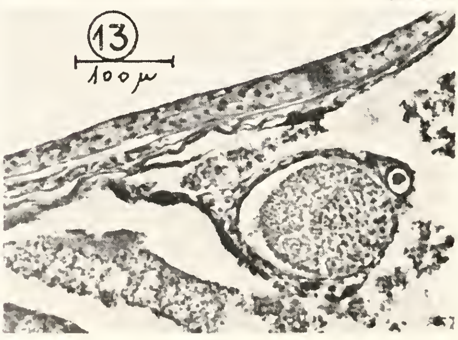












FIGURES 7–13.

The effects of methyl testosterone and stilbestrol diphosphate were assayed in concentrations ranging from 50 to 800 μg. per liter water; the other hormones were tested for their effects only at the concentration of 1000 μg. per liter.

Cultures treated with androgens

The androgens examined had, even at the highest concentrations examined (1.0 mg. per liter), only variable effects on gonadal differentiation. While there were individuals with almost involuted gonads (Fig. 9), others showed only small histological deviations from control glands (Fig. 8). The sex ratio was not changed by this treatment. The only clear-cut effect of androgens observed was a delay of several weeks in gonadal differentiation. The mortality in this group was minimal. The partial refractoriness of the gonads to androgens at the high concentrations employed is of interest, in view of positive results obtained by investigators with much lower concentrations of androgens: in *Phoxinus laevis* by Bullough (1940), in *Xiphophorus* by Regnier (1939), in *Lebistes* by Mohsen (1958) and by Miyamori (1961), and in the trout by Ashby (1957).

Cultures treated with stilbestrol diphosphate

The mortality of fish in concentrations of estrogens higher than 200 μg. per liter, from the third week onward, was considerable. At the concentrations 400, 800 and 1000 μg. per liter, only a few individuals remained alive until the end of the treatment. At autopsy these fishes showed heavy internal hemorrhages.

In the concentrations of 50 and 100 μg. estrogen per liter, the mortality was minimal. These concentrations had a powerful inhibitory effect on the gonad. In most cases the estrogen caused the elimination of all the germinal and stromal cells and left only the peritoneal lining (Fig. 10). At the higher concentrations (200 μg. per liter), the internal organs developed enormous vascular supply and the coelomic cavity was filled with a deeply chromophilic colloidal mass (Fig. 11). The tubules of the nephrons were enlarged. Since sex characters were indistinguishable, no distinction between genetic sexes could be made. The peritoneal lining was not hypertrophied or in any other way affected by this treatment. This is at variance with the observations on *Salmo trutta* by Ashby (1957), on *Xiphophorus helleri* by Friess (1933), on *Triton* by Gallien (1954) after estrogen treatment, and on *Ambystoma* by Mintz (1947) after testosterone treatment.

The sequence of gonadal destruction could be traced by observing the histologi-

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FIGURE 7. Ovary at 9 weeks.

FIGURE 8. Ovary at 10 weeks, 5 weeks in the hormone solution containing testosterone propionate, 800 μg. per liter.

FIGURE 9. Gonad at 10 weeks, 5 weeks in the androgen solution (testosterone propionate, 800 μg./L.), showing complete degeneration. Only the peritoneal lining is left.

FIGURE 10. Gonad at 9 weeks, 4 weeks in stilbestrol diphosphate, 100 μg. per liter, showing complete degeneration.

FIGURE 11. Intense hyperrhaemia of the liver at 9 weeks of age, 4 weeks in stilbestrol diphosphate, 200 μg. per liter.

FIGURE 12. Large blood vessels (BV) in the gonad, at the age of 8 weeks, 3 weeks in the solution of stilbestrol diphosphate, 200 μg./L.

FIGURE 13. A large blood vessel occupying the whole space of the gonad, at the age of 9 weeks, 4 weeks in stilbestrol diphosphate, 200 μg. per liter.

cal changes occurring at weekly intervals during the treatment. In the estrogen concentration of 50 μg. per liter, as well as in the 100 μg. per liter, two weeks of treatment sufficed to elicit first signs of gonadal destruction. The gonocytes contained pycnotic nuclei and were at this time at early stages of degeneration. The number of stroma cells was strongly diminshed. After three weeks in the estrogen solution, large blood vessels penetrated the gonads (Fig. 12). Gradually the blood vessels occupied the whole space of the gland. After four weeks of treatment, only the peritoneal lining of the gonads persisted (Fig. 13). The mechanism of this destruction of the gonad is, at present, not clear.

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SUMMARY

The normal differentiation of the gonads of *Tilapia aurea* is described. The process is similar to that described for other teleosteans and differs only in a few points. (a) The appearance of the first sign of the gonad is delayed and takes place 10 days after hatching. (b) The path of migration of the gonocytes to the gonadal anlagen in the anterior part of the fish is on the splanchnopleura. Only at the caudal quarter of the gland, the path of migration is the lateral part of the somatopleura.

Estrogens in concentrations of 50 and 100 μg. per liter of aquarium water, for a period of 3–4 weeks at the time of gonadal differentiation, bring about the destruction of the gonads of *Tilapia*. Androgens at a concentration as high as 1.0 mg. per liter have only a variable effect on the destruction of the gonad.

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EFFECT OF INHIBITORS ON ACTIVE TRANSPORT BY TURTLE INTESTINAL SEGMENTS[1]

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Intestinal absorption of sugars against a concentration gradient has been shown to occur in cold-blooded as well as warm-blooded animals (Csaky and Fernald, 1960; Fox, 1961a, 1961b; Lawrence, 1963; Musacchia *et al.*, 1964). The studies reported here used a number of inhibitors to reveal the energy source for glucose absorption by intestinal segments (*in vitro*) of the painted turtle, *Chrysemys picta*.

MATERIALS AND METHODS

Painted turtles (5–6″ shell length) of both sexes were obtained commercially. They were kept at room temperature during all seasons, and were fed pieces of horsemeat once each week. The animals were sacrificed by severing the spinal cord with a bone shears. Segments 6 cm. in length were dissected from the upper small intestine and sacs prepared using the method of Crane and Wilson (1958). This method calls for eversion of each segment so that the mucosal epithelium is "outside" and transport of materials is from the outer surface toward the inner.

The incubation medium consisted of Krebs-Ringer-bicarbonate (NaCl = 0.7%) plus 4.5 mg./100 ml. D-glucose (2.5×10^{-4} *M*), with or without inhibitor. One ml. of this solution was placed inside the sac (serosal side) and the preparation was immersed in 10 ml. of the same solution (mucosal side). A control segment and one or two experimental segments from the same animal were incubated simultaneously in a Dubnoff shaking water bath in separate Erlenmeyer flasks for 60 minutes at 30° C. This temperature was chosen because previous work with *Chrysemys picta* (Fox, 1961b) had shown the mucosal uptake and serosal accumulation of D-glucose to be at a maximum at 30° C. Air or nitrogen was bubbled through the medium on the mucosal side during incubation. After 60 minutes, 0.5-ml. samples of the mucosal and serosal solutions were analyzed for their glucose concentration, using the method of Nelson (1944) or the glucose oxidase reaction.

The following compounds at different concentrations were added to the incubation medium in separate experiments: potassium salt of monoiodoacetic acid (IAA), 2,4-dinitrophenol (DNP), phlorizin, disodium salt of malonic acid, sodium azide, sodium cyanide and ouabain. In one series of experiments nitrogen rather than air was bubbled through the mucosal fluid.

Each experimental run had its own control segment; mucosal uptake of D-glucose by the segments exposed to an inhibitor was compared to mucosal uptake by the control segment taken from the same animal. Segments were weighed and wet weights recorded at the end of each run. The amounts of glucose taken up

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TABLE I

*Inhibition (%) of active transport of D-glucose by isolated intestinal segments of Chrysemys picta**

Inhibitor	10^{-1} *M*	10^{-2} *M*	10^{-3} *M*	10^{-4} *M*	10^{-6} *M*	Number of segments used
Azide		0	0	0		32
Cyanide	70	60	15	0		51
Dinitrophenol	100	80	15	0		24
Iodoacetate		70	50	10	0	26
Malonate	70	50	0	0		27
Ouabain		90	75	50	90	23
Phlorizin			100	80	100	32
Nitrogen	No significant % of inhibition.					12

* All experiments were aerobic except those using nitrogen.

by the mucosa and added to the serosal side in micromoles per gram tissue (wet wt.) per hour were calculated and these data used to find the percentage of inhibition.

RESULTS

Table I shows the percentage of inhibition observed with different molar concentrations of the compounds tested. One hundred per cent inhibition indicates no net uptake of glucose from the mucosal solution; 0% inhibition indicates that uptake was statistically identical with controls. Phlorizin, iodoacetate and ouabain were potent inhibitors of glucose absorption at concentrations of 10^{-3} *M* or lower, while dinitrophenol, malonate, cyanide and azide were less effective.

When intestinal segments were exposed to 10^{-6} *M* IAA with nitrogen gas instead of air bubbled through the solution, mucosal uptake of glucose was completely inhibited (Table II). This is in contrast to the absence of any inhibitory effect in the presence of IAA (10^{-6} *M*) under aerobic conditions (Table I).

TABLE II

Effect of IAA and nitrogen gas on active transport of D-glucose (initial concentration on both sides = 4.5 mg./100 ml.) by intestinal segments (in vitro) of Chrysemys picta

	μMoles D-glucose/gm. tissue (wet wt.)/hr.		Number of segments used
	Mucosal solution	Serosal solution	
Air bubbled through mucosal solution	−2.2±0.51*	+0.08±0.01	12
Nitrogen gas bubbled through mucosal solution	−2.5±0.38*	+0.20±0.12	8
IAA (1 × 10^{-6} *M*) + Air	−2.0±0.61*	+0.10±0.01	8
IAA (1 × 10^{-6} *M*) + Nitrogen	+1.3±0.29*	+0.75±0.15	8

* Mean and the standard error of the mean.

DISCUSSION

Inhibitors are known to act at different sites to block the active transport of sugars by intestinal tissue (Darlington and Quastel, 1953; Crane, 1960).

Phlorizin

Phlorizin inhibits glucose transport systems in both kidney tubules and intestinal tissues (Nakazawa, 1922; Jolliffe *et al.,* 1932; Jervis *et al.*, 1956). At a concentration of 10^{-6} *M*, phlorizin prevents entry of glucose into the mucosal cells, while higher concentrations interfere with the cellular oxidation of glucose (Parsons *et al.*, 1958; Ponz and Balasch, 1964). According to Alvarado and Crane (1962) phlorizin blocks glucose absorption by competitive inhibition for a "transport site" on a carrier in the brush border.

The studies reported here demonstrate transport inhibition by phlorizin at a concentration of 10^{-6} *M*, as well as the more generalized effect on cell metabolism at concentrations of 10^{-4} *M* and higher. This suggests that turtle mucosal cells contain a carrier or transport site similar to that of higher vertebrates.

Iodoacetate

At low concentrations, iodoacetate is apparently selective in its inhibition of the enzymes of glycolysis, specifically triosephosphate dehydrogenase and phosphofructokinase (Webb, 1963). Working with iodoacetate-poisoned turtles (*Sternothoerus minor*), Belkin (1962) demonstrated a greatly decreased tolerance to anoxia, and concluded that glycolysis is a necessary source of metabolic energy for animals under these conditions. In the present study, when air was replaced by nitrogen during incubation of IAA-treated segments, total inhibition was observed (Table II). These data may be interpreted as further evidence that the turtle can use energy derived from anaerobic glycolysis to drive its transport mechanism.

2,4-Dinitrophenol

Compounds such as 2,4-dinitrophenol are known to depress formation of ATP by dissociating the reactions of phosphorylation from those of electron transfer. This uncoupling phenomenon may be demonstrated at a DNP concentration (10^{-4} *M*) which is ineffective in bringing about any general respiratory inhibition. DNP inhibits the active transport of glucose by rat intestinal segments at 10^{-4} *M* (Darlington and Quastel, 1953) but was found to be ineffective with our turtle preparations. The inhibitory effects at higher DNP concentrations (Table I) may be ascribed to the generalized effect on cell metabolism. We conclude that oxidative phosphorylation is not a necessary source of the energy for absorption of glucose against a concentration gradient by turtle intestinal segments.

Ouabain

A number of investigators (Weatherall, 1960; Csaky *et al.,* 1961; Parkinson, 1964) have shown that the cardiac glycosides exert an inhibitory effect on cellular transport of sodium and potassium ions. At concentrations of 10^{-5} *M* or less, the effect appears to be at some site on the cell membrane, but at 10^{-3} M or higher, these compounds serve to uncouple oxidative phosphorylation, and a specific effect on the sodium pump cannot be demonstrated. Table I shows an inhibitory effect of the glycoside ouabain at a concentration of 10^{-6} M. These data support the hypothesis of Crane *et al.* (1961), Crane, (1962) and Csaky (1963) that a sodium transport system is associated with sugar transport, and serve as further evidence of the basic similarity of transport mechanisms in all types of vertebrates.

Azide, cyanide and malonate

Azide and cyanide compounds act to inhibit metallo-enzymes such as cytochrome oxidase. Darlington and Quastel (1953) reported complete inhibition of active transport of glucose by rat intestine by 10^{-2} M concentrations of sodium azide and sodium cyanide. Table I shows that turtle intestinal absorption was only 60% inhibited by cyanide at this concentration and was insensitive to azide. These data reinforce the hypothesis that oxidative respiration *via* the cytochrome chain is not essential for intestinal active transport of glucose in *Chrysemys picta*. Neither does the Krebs cycle appear to be essential, since malonate caused only 50% inhibition even at a relatively high concentration (10^{-2} M).

Lowered oxygen tension

Several recent publications have documented the fact that turtles are highly tolerant of anoxic conditions such as those normally encountered by these animals in diving and during periods of cold torpor (Belkin, 1963; Klahr and Bricker, 1964; Musacchia *et al.*, 1964; Robin *et al.*, 1964). It appears that turtle cell respiration is able to shift from aerobic to anaerobic pathways in reaction to lowered intracellular oxygen tension or inhibition of the cytochrome system. In such situations the reactions of glycolysis become the prime source of metabolically useful energy.

The concept of turtle metabolism which emerges from these studies is one of flexibility and tolerance to conditions where oxygen is in short supply. We conclude that the turtle may use oxidative pathways but is partially, and at times entirely, dependent upon glycolysis for the energy required for intestinal active transport of sugars.

We acknowledge the assistance of Mary Kirkhoff, Shirley Maloney and Kathleen Fitzgerald in portions of this study.

SUMMARY

1. Isolated segments of turtle (*Chrysemys picta*) intestine were exposed to metabolic inhibitors and the effects on active transport of D-glucose (4.5 mg./100 ml.) were studied.

2. Phlorizin, iodoacetate and ouabain were potent inhibitors; 2,4-dinitrophenol, malonate, cyanide and azide were less effective.

3. No significant inhibition was observed when nitrogen replaced aeration of the segments, except when segments were simultaneously treated with iodoacetate.

4. The energy for the sugar transport mechanism in this animal appears to come from glycolysis.

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ION DISTRIBUTION AND TRANSPORT IN THE RED MARINE ALGA, GRACILARIA FOLIIFERA[1, 2]

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Marine algae were the object of many early studies of electrolyte permeability and transport (see Blinks, 1940; Osterhout, 1940). However, there have been few recent investigations of ionic relations in marine algae. In *Ulva* and *Hormorsira* independent and oppositely directly Na^+ and K^+ transport systems were proposed (Scott and Hayward, 1954, 1955; Bergquist, 1958, respectively). *Porphyra,* a non-vacuolated red alga, actively extruded Na^+ and passively accumulated K^+ (Eppley, 1958a, 1958b, 1959, personal communication). MacRobbie and Dainty (1958a, 1958b) demonstrated active efflux of Na^+ and active influx of Cl^- in *Rhodymenia,* a vacuolated red alga, and in *Nitellopsis,* a brackish water characean. *Halicystis* actively extruded Na^+ and actively absorbed Cl^-, whereas K^+ was in electrochemical equilibrium between vacuole and sea water (Blount and Levedahl, 1960).

In some, but not all, of these studies membrane potential measurements were made in conjunction with ion distribution and flux measurements. As pointed out by Ussing (1960) and Dainty (1962), this information is needed to determine exactly which ions are actively transported, *i.e.,* moved against an electrochemical potential gradient.

This is a study of alkali and halide ion transport in *Gracilaria foliifera,* a red marine alga. The major monovalent ions, Na^+, K^+ and Cl^-, are included, as well as the less abundant ions, Rb^+, Cs^+, Br^- and I^-. The latter were included for comparative purposes and also because of their radiobiological importance as components of fallout and nuclear reactor wastes. A primary objective of this work was to determine which of these ions are actively transported by cells of *Gracilaria.* The major criterion for active transport was the existence of an appreciable difference between the calculated Nernst equilibrium potential for each ion and the measured vacuole potential. The effects of some environmental factors, mainly light and anaerobiosis, on ion fluxes and their rate coefficients were studied also.

Gracilaria foliifera is a red seaweed common on the North Carolina Coast. Its slightly compressed branches are composed of large vacuolated cells, 100–150 μ in diameter, surrounded by several layers of small cells, 10–20 μ in diameter, and a tough outer cuticle. Visual estimates indicate that the vacuoles of the large cells comprise more than half the tissue volume. Most of the remaining volume, as shown below, is extracellular space and some impermeable material. The protoplasm comprises only a small fraction of the tissue.

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METHODS

Experimental procedure

Gracilaria was collected from rock jetties near the Beaufort harbor inlet and placed in 40-liter plastic tanks containing aerated sea water. Constant illumination was provided by two 40-watt "daylight" fluorescent tubes placed about 60 cm. from the seaweed. Water temperature ranged from 15° to 20° C. The alga was kept under these conditions for several months without apparent deterioration.

Experiments were conducted with small branches of the seaweed, 3–4 cm. and 10–30 mg. each, contained in 2-liter flasks of aerated medium. The basic medium was natural sea water (salinity 32–35‰), buffered at pH 7.8–7.9 with tris(hydroxymethyl)aminomethane (10 m*M*) and containing added $NaNO_3$ (100 μM) and NaH_2PO_4 (50 μM). The test solutions were changed daily. Experiments were conducted either in the dark (flasks painted black) or under constant illumination of 8500 ± 1000 lux provided by six 40-watt "daylight" tubes, 20–30 cm. from the flasks. The experimental temperature was 24 ± 1° C. Under these conditions the growth rate in the light was 0.5–1.0%/hr., whereas no detectable growth occurred in the dark.

Sodium and potassium were measured by flame photometry (Beckman DU spectrophotometer with flame attachment),[3] and chloride was measured with an Aminco-Cotlove automatic chloride titrator. Sodium and potassium were extracted by soaking tissues overnight in HNO_3 (1.0 or 0.2 N). Chloride was extracted either by this method or with hot distilled water. Similar results were obtained with both methods.

Cesium, Rb^+ and I^- were not measured chemically. The relative intracellular concentration of these elements was determined by radioactivity measurements, and is expressed as a concentration ratio (c.p.m./g. cell water):(c.p.m./ml. environment). The natural concentrations of Cs^+ and Rb^+ in sea water are about 0.04 μeq./l. and 1.4 μeq./l., respectively (Smales and Salmon, 1955). The total iodine concentration in sea water is about 0.4 μeq./l. (Barkely and Thompson, 1960; Sugawara *et al.*, 1962).

Gamma-emitting radioisotopes (Cs^{137}, Rb^{86}, K^{42}, Br^{82} and Na^{22}) were measured in a 3-inch well-type scintillation detector connected to a single-channel analyzer (Baird-Atomic Model 510). Tissues were removed periodically from the flasks, blotted with absorbent tissue, wrapped in plastic, counted, weighed and returned to their solutions. Carbon-14 and Cl^{36} were measured in an end-window gas flow detector with a low-background attachment (Nuclear Chicago Model C-115). Several 1-ml. aliquots of neutralized tissue extract or medium were dried in planchets for counting.

Radioisotopes were purchased from the Oak Ridge National Laboratory, Oak Ridge, Tenn. (K^{42}, Rb^{86}, Cs^{137}, Cl^{36}, Br^{82} and I^{131}); Nuclear Science and Engineering, Pittsburgh, Pa. (Na^{22}); and Calbiochem, Los Angeles, Cal. (C^{14}-mannitol and C^{14}-sucrose). Tracers were either "carrier-free" or of sufficiently high specific activity so that the natural concentration of the element was increased less than 3%.

The electrical potential of the vacuole was measured with glass microcapillary electrodes and a high-impedance voltmeter (Keithley Model 600A). Pipettes with tip diameters of 4–8 μ were drawn from 1 mm. Pyrex tubing and filled with

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[3] Trade names referred to in this publication do not imply endorsement.

0.5 M KCl by boiling under reduced pressure. The pipette was connected to the voltmeter by a KCl-agar bridge and calomel half-cell. An identical half-cell and sea-water-agar bridge completed the circuit, making contact with the bathing sea water which was taken as ground. The electrodes had tip potentials of less than 2 mv. in either sea water or 0.5 M KCl.

To measure the vacuole potential, a branch was held rigidly with waterproof putty in a glass dish of sea water under a dissecting microscope. The cells were exposed by an oblique cross-sectional cut, and the puncture was made with the aid of a micromanipulator. Ten to 20 minutes were usually required for the potential to reach a stable value. After this, only potentials which were stable (±1 mv.) for at least 30 minutes were recorded.

Estimation of ion fluxes and rate coefficients

The difficulties of estimating ion fluxes and their rate constants in a heterogeneous cell population were emphasized by MacRobbie and Dainty (1958a) in their study of Na^+ and K^+ fluxes in the seaweed *Rhodymenia*. Since their admonitions apply also to the tissues of *Gracilaria*, fluxes were estimated in terms of cell water rather than in terms of membrane area. Also, rate constants, or rate coefficients (since they varied with time), were measured during successive time intervals and averaged.

Unidirectional fluxes were estimated for the ions in which specific activities were known (Na^+, K^+ and Cl^-), using the relations

$$M_i = \frac{C_i^*}{t} \frac{C_o}{C_o^*}$$

$$M_o = k_o C_i$$

where M_i and M_o are the initial influx and efflux in meq. (kg. cell water)$^{-1}$ hours^{-1}; C_o^* and C_o are the labeled ion and total ion concentrations outside; C_i is the total intracellular ion concentration; C_i^* is the labeled ion concentration inside the cells at time, t; and k_o is the rate of coefficient for efflux, calculated as shown below. Influx values were calculated by using data from the early portion of isotope uptake experiments, when the efflux of labeled ions was considered negligible.

Rate coefficients for isotope influx (k_i) and efflux (k_o) were calculated from the equations

$$k_i = \frac{2.3}{t} \log \frac{C_{i,oo}^*}{C_{i,oo}^* - C_i^*}$$

$$k_o = \frac{2.3}{t} \log \frac{C_{i,0}^*}{C_i^*}$$

where $C_{i,oo}^*$ is the intracellular labeled ion concentration at isotopic equilibrium; $C_{i,0}^*$ is the initial intracellular labeled ion concentration; and C_i^* and t have the same meanings as before. The two rate coefficients, one calculated from isotope influx data and the other from isotope efflux data, provided a means of comparing the unidirectional fluxes of ions for which specific activities were not known (Cs^+, Rb^+, Br^- and I^-).

These equations are similar to those used recently by House (1963). They are derived from standard flux equations, treating the cells as a single compartment (see, for example, Sheppard, 1962). In experiments involving tracer efflux into a large nonlabeled environment, the usual plot of log activity remaining *vs.* time was often nearly linear, indicating fairly good agreement with predicted single-compartment behavior.

RESULTS

Tissue water, extracellular space and cell water

To estimate intracellular ion concentrations, the amounts of tissue water and extracellular space must be known. Tissue water, measured by comparing the fresh weight (after blotting with absorbent tissue) with the dry weight (24 hr. at 105° C.), was found to be 89 ± 1% (6). (Results are quoted in the sequence: mean, standard error and [in parentheses] number of measurements.)

Extracellular space (mainly the free space in the cell walls and between the cells) was measured by soaking tissues for 2–3 hours in sea water containing C^{14}-mannitol or C^{14}-sucrose. Tissues were then blotted, weighed, and washed in 50 ml. unlabeled sea water. From the amount of C^{14} which diffused from the tissues to the environment (loss was complete after 15–30 minutes), it was calculated that the tissue contained 19 ± 2% (5) sucrose or mannitol space. The same method applied to tissues freshly killed (by immersing for 10 seconds in sea water at 90° C.) gave a value of 91 ± 2% free space, suggesting that about 9% of the tissue was impermeable to the labeled sugar. Although the agreement may be fortuitous, the latter value is close to the 11% dry weight found above.

Extracellular space also was checked by using Na^{22}, Cl^{36} and Br^{82}. The amounts of these ions washed out in 8–10 minutes with either 0.6 *M* sucrose or unlabeled sea water agreed with the amounts predicted on the basis of 19% sucrose or mannitol space. This supports the assumptions that ion concentrations in the extracellular space are similar to those in sea water and that exchange of intracellular ions during a 10-minute wash in sucrose or sea water is insignificant. The other ions studied (Rb^+, Cs^+, K^+ and I^-) were predominantly intracellular, so that some extracellular adsorption (although there was little indication of this) would not appreciably affect the estimated intracellular ion concentrations.

Cell water was estimated, using the above figures, to comprise 69–71% of the tissue (*i.e.*, the difference between per cent tissue water and per cent extracellular space). Therefore, the intracellular ion concentrations were calculated as 100/70 = 1.4 times the tissue ion concentration, either following a 10-minute wash in 0.6 *M* sucrose or after subtracting the estimated concentration of ions in the extracellular space.

Electrical potential of the vacuole

The vacuole potential, measured by means of microcapillary electrodes, was −81 ± 2 mv. (12). The highest recorded potential was −92 mv. The measurements were usually made after 12–48 hours of illumination, although several hours of darkness had no obvious effect on the potential.

Ion distribution and equilibrium potentials

The concentrations of monovalent ions in *Gracilaria* and in sea water are shown in Table I. Concentration ratios (C_i/C_o) are based either on chemical and radioactivity measurements (Na^+, K^+ and Cl^-) or on radioactivity measurements alone (Rb^+, Cs^+, Br^- and I^-). The C_i/C_o values obtained in tracer experiments represent the steady-state levels attained in the light (after 10 hours to 9 days exposure, depending upon the ion). Chemical measurements of Na^+, K^+ and Cl^- were made after about 48 hours illumination.

Potassium, the principal intracellular cation in *Gracilaria,* was concentrated about 60-fold (Table I). Sodium, in contrast, was excluded by a factor of about seven. Chloride, the principal intracellular anion, was present at over 60% of the concentrations of Na^+ plus K^+. About 70% of the tissue Na^+ and 25% of the tissue Cl^- were extracellular. Rubidium, Cs^+ and I^- were all highly concentrated by cells of *Gracilaria,* although they were of little quantitative importance compared to Na^+, K^+ and Cl^-.

TABLE I

*Concentrations and equilibrium potentials of monovalent ions in Gracilaria and in sea water**, **

Ion	Sea water (meq./l.) (C_o)	*Gracilaria*		Concentration ratio (C_i/C_o)	Equilibrium potential (mv.) (E_j)
		(meq./kg. fresh wt.)	(meq./kg. cell H_2O) (C_i)		
Na^+	471 ± 11 (11)	161	66.3 ± 4.6 (13)	0.14 ± 0.01 (11)	+50
K^+	11.0 ± 0.2 (9)	488	680 ± 12 (12)	61.8 ± 1.1 (9)	−105
Rb^+	1.4×10^{-3}	—	—	148 ± 5 (19)	−127
Cs^+	0.04×10^{-3}	—	—	28.6 ± 2.5 (8)	−85
Cl^-	532 ± 3 (8)	436	462 ± 17 (8)	0.87 ± 0.03 (8)	−3.6
Br^-	0.81	—	—	0.57 ± 0.03 (4)	−14
I^-	0.4×10^{-3}	—	—	150 ± 7 (4)	+128

* Sea water concentrations of Rb^+, Cs^+, Br^-, and I^- are approximate, based on published values. Concentration ratios for these ions, based on radioactivity measurements, are more accurate, however.

** Results expressed as mean, S.E. and (in parentheses) number of measurements.

An equilibrium potential may be calculated for each ion, using an approximation of the Nernst equation

$$E_j = \frac{-2.3\ RT}{z_j F} \log \frac{C_i}{C_o}$$

where E_j is the electrical potential across a membrane separating an ion at two concentrations, C_i and C_o. The constants R, T, and F have their usual meanings, and z_j is the algebraic valency of the ion, j. An appreciable difference between the vacuole potential (E) and the equilibrium potential (E_j) indicates that the ion is not in passive flux equilibrium across the membrane and suggests, therefore, active transport of that ion (see Dainty, 1962).

Most of the calculated equilibrium potentials shown in Table I do not agree

TABLE II

Rate coefficients for isotope fluxes in Gracilaria. Effects of light and darkness, ***

Ion	Rate coeff. of isotope influx (k_i)	Rate coeff. of isotope efflux (k_o)	
	Light	Light	Dark
Na^+	30 ± 1 (9)	80 ± 9 (8)	14 ± 4 (6)
K^+	20 ± 2 (6)	9.8 ± 0.5 (7)	0.47 ± 0.09 (5)
Rb^+	1.6 ± 0.2 (11)	0.67 ± 0.1 (13)	0.09 ± 0.02 (4)
Cs^+	1.9 ± 0.2 (9)	0.46 ± 0.05 (14)	0.22 ± 0.08 (4)
Cl^-	1.8 ± 0.1 (9)	0.58 ± 0.1 (6)	0.14 ± 0.03 (5)
Br^-	0.81 ± 0.08 (7)	0.88 ± 0.08 (5)	0.35 ± 0.05 (5)
I^-	2.5 ± 0.3 (5)	0.55 ± 0.04 (3)	0.21 ± 0.06 (3)

* Units are $10^2 hr.^{-1}$.

** Results expressed as mean, S.E. and (in parentheses) number of measurements.

with the measured vacuole potential of −81 mv. Of the major ions, Na^+ is farthest from electrochemical equilibrium, both electrical and chemical gradients being directed inward. This suggests that Na^+ is actively transported out of the cells. Potassium, Rb^+, Cl^- and Br^- are all concentrated inside at considerably higher levels than would be predicted from the vacuole potential of −81 mv. Cesium is close to electrochemical equilibrium, and the status of I^- is uncertain since, as is shown below, there is appreciable intracellular binding of I^-. Thus K^+, Rb^+, Cl^- and Br^- appear to be actively absorbed by cells of *Gracilaria,* and Na^+ appears to be actively extruded.

To justify using the Nernst equation, major shifts in ion distribution should not occur under the experimental conditions. To check this, tissues were illuminated for 48 hours, then kept for 5 days in the dark, and also under nitrogen in the dark. Under these conditions the intracellular K^+ and Cl^- concentrations decreased less than 10%. Similarly, there was a net loss of about 5% of the radioactive Rb^+ and Cs^+ from maximally labeled tissues which were kept for 5 days under the same two conditions, in a constant radioactive environment. Intracellular Na^+ decreased in the dark from about 70 to 50 meq./kg. cell water, and increased under nitrogen from about 70 to 90 meq./kg. cell water. The net ion movements were slow compared to the unidirectional fluxes given below. Thus, under the conditions of this study, intracellular ion concentrations remained approximately constant.

TABLE III

Ion fluxes in Gracilaria. Effects of light and darkness, ***

Ion	Influx (M_i)		Efflux (M_o)	
	Light	Dark	Light	Dark
Na^+	18 ± 2 (5)	2.1 ± 0.3 (4)	54 ± 6 (8)	8.8 ± 2.5 (6)
K^+	101 ± 2 (8)	9.1 ± 3.1 (6)	66 ± 4 (7)	3.2 ± 0.5 (5)
Cl^-	2.0 ± 0.5 (6)	1.0 ± 0.4 (5)	2.7 ± 0.5 (6)	0.62 ± 0.2 (5)

* Units are meq. (kg. cell water)$^{-1}$hr.$^{-1}$.

** Results expressed as mean, S.E. and (in parentheses) number of measurements.

Another requirement for calculating equilibrium potentials is that intracellular ions be in solution rather than adsorbed or bound to cytoplasmic sites. In *Gracilaria* intracellular ion binding was expected to be small, since the cytoplasm comprises only a small fraction of the large vacuolated cells. With K^{42}, Rb^{86} and Cs^{137} this was substantiated by the observation that maximally labeled tissues, when killed and held in the same radioactive environment, lost virtually all of their radioactivity

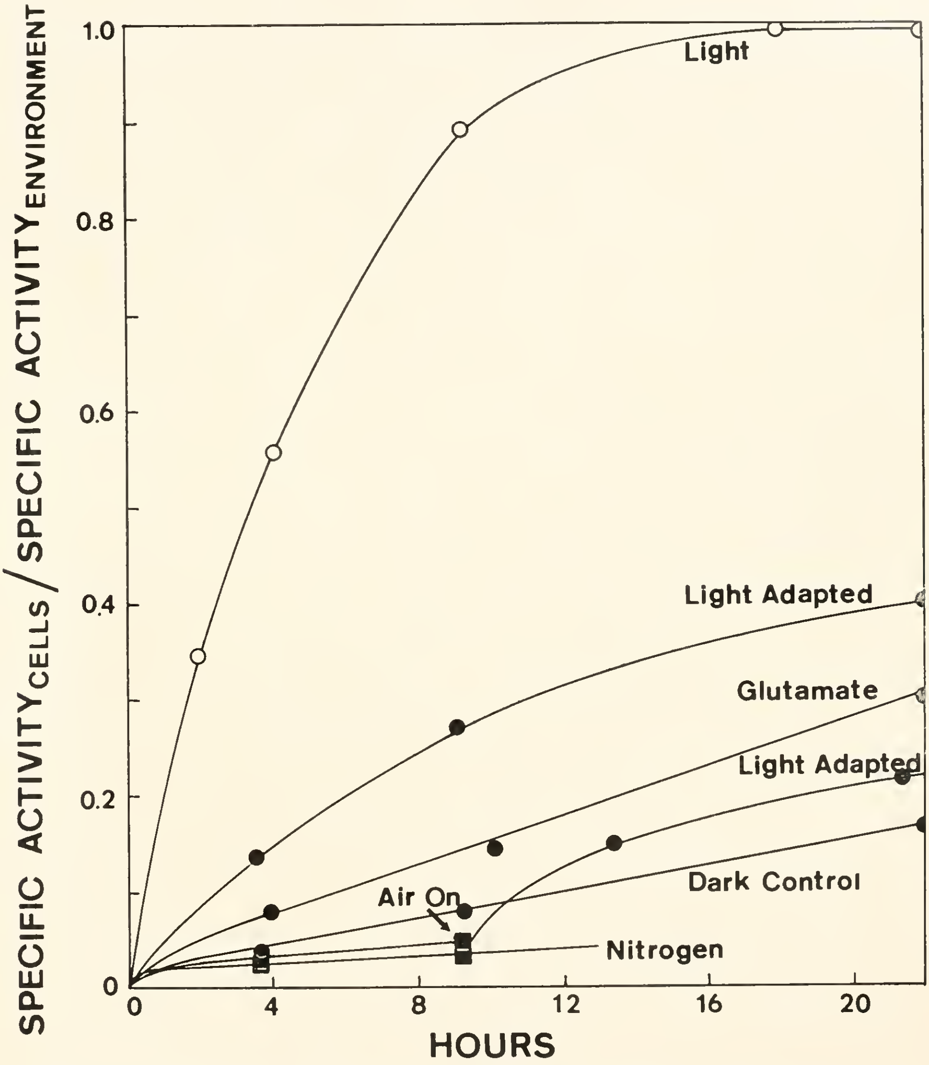

FIGURE 1. Potassium-42 uptake by cells of *Gracilaria*. Effects of light (8500 lux) (○), darkness (●), anaerobiosis (nitrogen) (■), glutamate (50 m*M*), and previous exposure to light (20 hours at 8500 lux). Arrow indicates the addition of air to tissues previously exposed to light, then placed in a K^{42} environment under nitrogen. Each point represents a measurement of 30–40 branches.

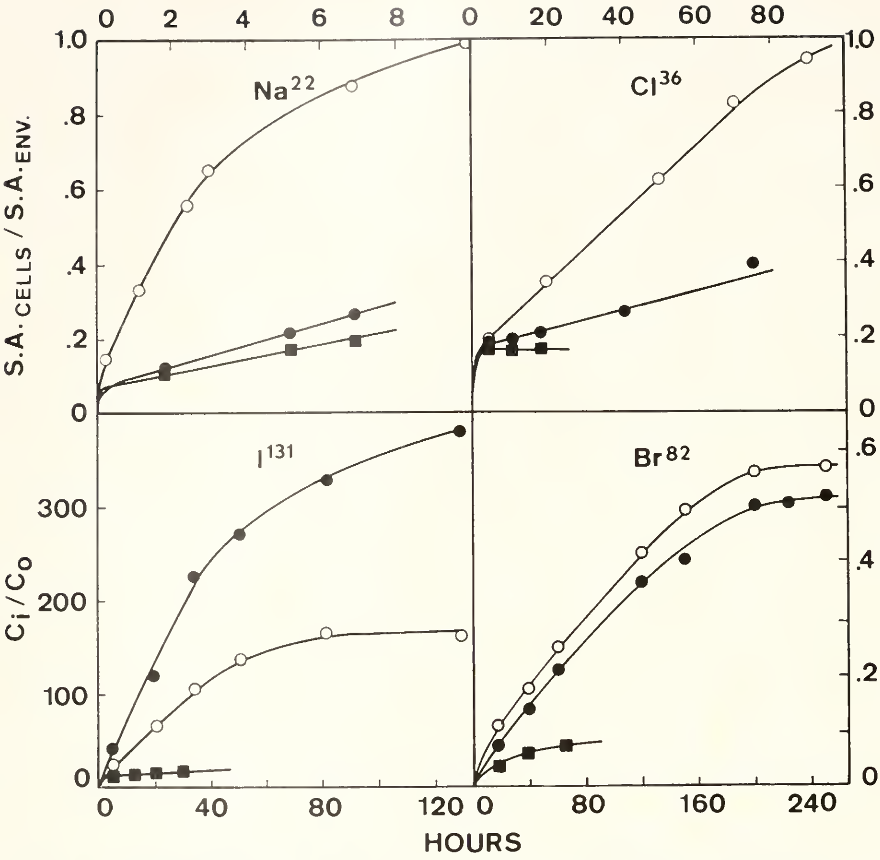

FIGURE 2. Uptake of Na^{22}, Cl^{36}, Br^{82} and I^{131} by cells of *Gracilaria*. Effects of light (○), darkness (●), and anaerobiosis (■). Radioactivity of cells expressed as a concentration ratio (C_i/C_o) or as the ratio of the specific activity (S.A.) of the cells to the S.A. of the environment. Each point represents a measurement of 15–40 branches. Note the differences in time and radioactivity scales.

in 20–30 minutes. Possible adsorption of the trace elements Rb$^+$ and Cs$^+$ was checked also by exposing the tissues to a wide range of low concentrations (0.01–100 μeq./l.). The uptake data, plotted as a Freundlich adsorption isotherm (Freundlich, 1926), gave linear log C_i *vs.* log C_o curves with a slope of 1.0 (Gutknecht, 1965). In other words, uptake over this range of concentrations was exactly proportional to external concentration, which is not likely in an adsorption-type reaction (Briggs, Hope and Robertson, 1961).

Iodide-131, in contrast to the other ions studied, was at least partly bound. Maximally labeled tissues, when killed and held in their original radioactive environment, retained about 25% of their radioactivity. Interestingly, tissues exposed to I^{131} in the dark contained a much larger amount of bound radioactivity. This difference was reflected in a higher rate of uptake as well as a higher C_i/C_o of 416 ± 20 (4), compared to the value of 150 found in the light (Table I).

All of the Na$^+$, K$^+$ and Cl$^-$ in *Gracilaria* appeared to be exchangeable in the light. The specific activities of the tissues and environment became equal after exposure periods of about 10 hours for Na^{22}, 20 hours for K^{42} and 100 hours for Cl^{36}. In general, all the ions except I^- appeared to be unbound and exchangeable and are no doubt largely in solution in the vacuolar sap. It is not likely, therefore, that ion binding plays an important role in determining the steady-state distribution of ions in *Gracilaria.*

Ion fluxes and rate coefficients

Ion fluxes were measured in the light (8500 lux), dark and under anaerobic conditions (continuous bubbling with commercial nitrogen in the dark). The effects of previous exposure to light, as well as the effects of exogenous substrate

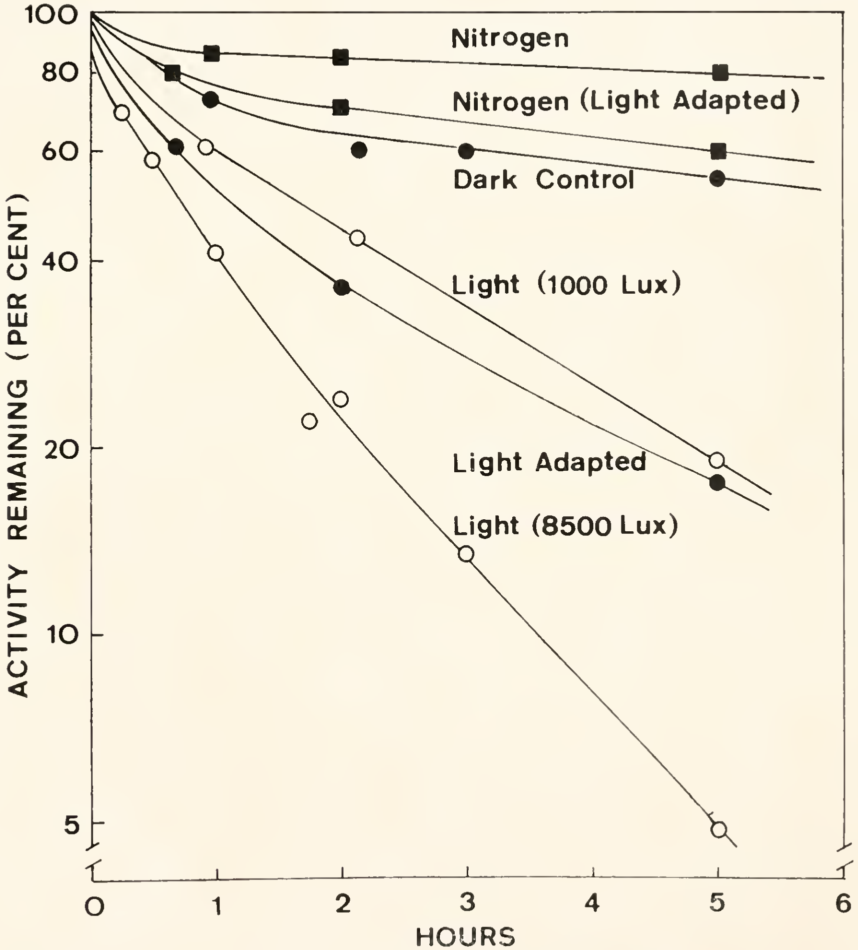

FIGURE 3. Loss of intracellular Na^{22} from *Gracilaria.* Effects of light (8500 lux to 1000 lux) (○), darkness (●), previous exposure to light (48 hours at 8500 lux), anaerobiosis (■), and anaerobiosis after previous exposure to light. Each point represents a measurement of 30–40 branches.

(sodium glutamate, 50 *mM*), were studied in some experiments. Unless otherwise indicated, tissues were adapted for 36–48 hours in either the light or dark, depending upon the subsequent experimental conditions. This conditioning was important since the effect of light on ion movements persisted during 10–20 hours of subsequent darkness.

Average fluxes and rate coefficients were calculated as described, and are listed in Tables II and III. Figures 1–4 show graphically the movements of labeled ions under various environmental conditions. The exchange of extra-

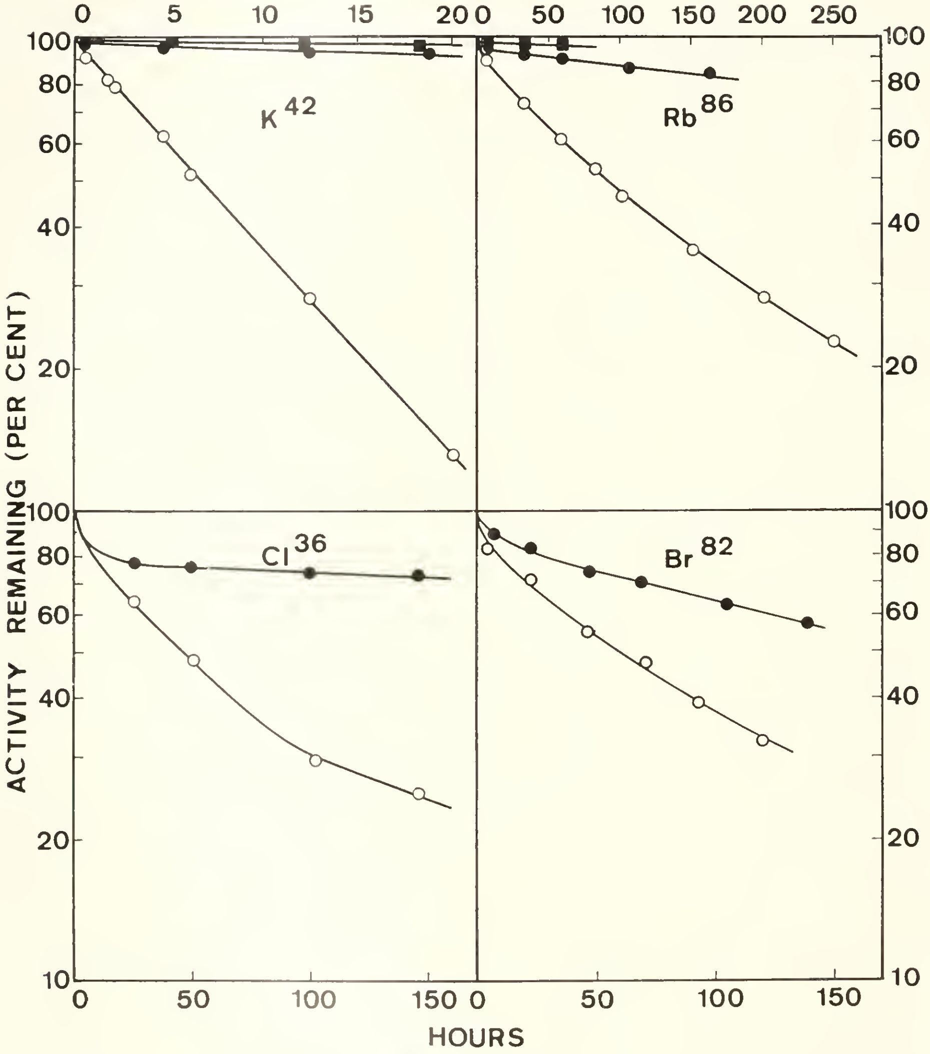

FIGURE 4. Loss of intracellular K^{42}, Rb^{86}, Cl^{36} and Br^{82} from *Gracilaria*. Effects of light (○), darkness (●), and anaerobiosis (■). Each point represents a measurement of 15–40 branches. Note the differences in time scales.

cellular ions is not included in either the figures or the tables, since the tissues were washed before counting. Nevertheless, a small and relatively fast intracellular compartment, or "shoulder," was often evident in both isotope uptake and loss experiments (see Figs. 1–4). This fast compartment also was excluded from the calculations of ion fluxes and rate coefficients. This compartment was generally less sensitive to environmental conditions than was the large slower compartment (presumably the vacuoles of the large cells).

Light promoted both influx and efflux of cations, as well as the efflux of anions (Figs. 1–4, Tables II and III). The effect of light on anion influx was variable, *i.e.*, Cl^- uptake was promoted by light, Br^- uptake was only slightly affected, and I^- uptake was depressed (Fig. 2). Anaerobiosis depressed the influx and efflux of cations and the influx of anions by factors ranging from 0.1 to 0.8, compared to the dark controls (Figs. 1–4).

Previous exposure to light had a temporary stimulating effect on K^+ influx and Na^+ efflux (Figs. 1 and 3). However, light-adapted cells placed in a radioactive environment under nitrogen did not show the temporary stimulation of the K^+ or Na^+ flux unless air was provided (Figs. 1 and 3). In the dark, glutamate (50 m*M*) increased the K^+ and Na^+ influx 2–3 times, compared to controls.

In general, the movements of Na^+, K^+, Rb^+ and Cs^+ were affected in a qualitatively similar manner by each of the environmental conditions shown in Figure 1. The major difference was that the rate coefficients for isotope influx and efflux decreased markedly in the order $Na^+ > K^+ > Rb^+, Cs^+$ (Table III).

DISCUSSION

All of the major ions (Na^+, K^+ and Cl^-) appear to be actively transported in *Gracilaria*, as indicated by the large differences between the vacuole potential (−81 mv.) and the equilibrium potentials shown in Table I. Sodium, as in most plant and animal cells, is farthest from electrochemical equilibrium ($E - E_{Na} = -131$ mv.), and is extruded against both electrical and chemical gradients. Active Cl^- absorption is indicated ($E - E_{Cl} = -77$ mv.), which is in contrast to most animal, but not plant, cells (see Dainty, 1962). Potassium, which is usually close to electrochemical equilibrium in both plant and animal cells, appears also to be actively absorbed ($E - E_K = 24$ mv.).

One of the assumptions on which the above interpretations rest was that the influx and efflux of an ion are equal. Although this was approximately true, there were some apparent deviations (Tables II and III). These may be due to net ion uptake with growth, as well as to errors involved in estimating fluxes and rate coefficients in a heterogeneous cell population. An alternative value, however, for the predicted vacuole potential, E_j, can be calculated by using the Ussing-Teorell flux-ratio equation (see Dainty, 1962, p. 384). The values for E_j obtained with this equation make even more stringent the requirements for active transport of most of the ions. That is, the $E - E_j$ values are larger than those obtained using the Nernst equilibrium potentials. The only exceptions were that $E - E_{Cl}$ decreased from −77 to −70, and $E - E_{Br}$ decreased from −67 to −65.

A second assumption was that selective binding of ions does not appreciably alter the estimated intracellular concentrations, especially of K^+ and Rb^+. (Any intracellular binding of Na^+ would make the requirements for active Na^+ extrusion even more stringent.) This was shown to be unlikely, since killed cells quickly lost all of their accumulated K^+ and Rb^+, and since (in the case of Rb^+) uptake was exactly proportional to external concentration over a wide range of low concentrations.

The third possible error could arise from a large difference between the cytoplasmic and vacuolar ion concentrations or electrical potentials. The main potential drop in plant cells, however, appears to be at the plasmalemma rather than at the tonoplast (see Dainty, 1962; Findlay and Hope, 1964; Spanswick and Williams, 1964). Also, since the vacuole:cytoplasm ratio in *Gracilaria* is high, it is unlikely that moderate differences between the ion concentrations in the cytoplasm and vacuole would appreciably change the calculated $E - E_j$ values for these ions.

Finally, the large differences between the C_i/C_o values for K^+, Rb^+ and Cs^+ (Table I) provide independent evidence for the involvement of an active process in the distribution of these ions. Otherwise the C_i/C_o values for these ions would be similar, regardless of the membrane potential. Once again, the absence of selective intracellular ion binding is an important requirement (see Relman *et al.*, 1957).

The present results may be compared with MacRobbie and Dainty's (1958a) study of Na^+ and K^+ fluxes in the seaweed, *Rhodymenia*. Their findings of an active Na^+ efflux and active K^+ influx were similar to those presented here. One exception was their observation that light had only a small effect on Na^+ fluxes, suggesting that Na^+ transport was more closely related to respiration than to photosynthesis. Their light intensity, however, was about 25% of that used in the present study. As shown in Figure 3, Na^+ efflux decreased by a factor of about 0.65 when the light intensity was reduced from about 8500 lux to about 1000 lux. Also, as shown in Figures 1 and 3, the effects of light on ion movements might be obscured if the cells used for measuring fluxes in the dark were not previously adapted to darkness.

A stimulating effect of light on ion transport in plant cells has been reported often, although the mechanism of light stimulation is not clear (see Briggs, Hope and Robertson, 1961; Dainty, 1964). In *Nitella* it was recently suggested that active K^+ uptake is supported by light energy through the utilization of ATP produced in photophosphorylation, whereas active Cl^- uptake is directly linked to light-driven electron-transfer reactions associated with oxygen evolution in photosynthesis (MacRobbie, 1965). The present observations on *Gracilaria* do little to clarify the way in which light promotes active ion fluxes (primarily Na^+ efflux and K^+ and Cl^- influx). With respect to the cation fluxes, the stimulating effects of previous exposure to light were inhibited by anaerobiosis (Figs. 1 and 3). If these fluxes were ATP-dependent, however, and ATP was synthesized in the light, then one might expect that previous exposure to light would stimulate the fluxes in nitrogen as well as in air.

The variable effect of light on anion uptake was an unexpected finding. Chloride uptake was promoted by light, Br^- uptake was only slightly affected, and I^- uptake was depressed in the light (Fig. 2). In contrast, Br^- uptake by *Nitella* was stimulated by light to a greater extent than was Cl^- uptake (MacRobbie, 1962). The depressing effect of light on I^- uptake was found to be even greater in three other seaweeds, *Ulva lactuca*, *Fucus vesiculosus* and *Hypnea musciformis*, than in *Gracilaria* (unpublished data). This appeared in every case to be due to a larger amount of bound I^{131} in tissues which absorbed I^{131} in the dark. Previous studies (see Shaw, 1962) suggest that in marine algae most of the intracellular iodine exists as iodide, although it may enter the cell in another form, thus raising doubts about the active transport of iodide, *per se*. Evidence from this and earlier studies, however, indicates that iodine is absorbed by an energy-dependent, if not, strictly speaking, an active transport process.

The passive ion fluxes, mainly Na^+ influx and K^+, Rb^+, Cs^+, Cl^-, Br^-, and I^- efflux, were all stimulated by light (Figs. 2 and 4, Tables II and III). It is possible that light simply increased the passive permeability to the anions, since the rate coefficients for Cl^-, Br^- and I^- efflux were similar and their hydrated ion sizes are similar (see Briggs, Hope and Robertson, 1961). The mechanism of passive cation fluxes, however, may be different, *i.e.*, not simple diffusion. This is because the rate coefficients of efflux were in the order $K^+ > Rb^+ > Cs^+$, which is not consistent with predictions based on size or mobility of the hydrated ions (see Ussing, 1960; Briggs, Hope and Robertson, 1961). Thus an important part of the K^+ efflux in *Gracilaria* probably occurs by a mediated, but not active, process—possibly exchange diffusion. This argument may be extended to include Na^+, since the hydrated Na^+ ion is larger than K^+. A similar suggestion was made by MacRobbie and Dainty (1958a) for *Rhodymenia*, based on calculations of an unusually high energy requirement for supporting active Na^+ efflux. Their suggestion would be applicable also to Na^+ efflux in *Gracilaria*.

The ability of *Gracilaria* to maintain its high intracellular K:Na ratio in the absence of both light and oxygen is in contrast to many higher plant and animal cells, as well as some other marine algae. *Ulva lactuca* and *Fucus vesiculosus*, for example, were unable to maintain their normal high K:Na ratio under anaerobic conditions, losing over 50% of their intracellular K^+ in 24 hours (unpublished data). In *Fucus* the loss of K^+ was irreversible after 48 hours. Interestingly, both these seaweeds are highly resistant to extremes of temperature and dehydration (Kanwisher, 1957). *Porphyra* also has been found to lose K^+ and gain Na^+ when exposed to nitrogen (Eppley, 1958a).

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SUMMARY

1. Distribution and transport of alkali and halide ions were studied in *Gracilaria foliifera*, a red marine alga. Ion concentrations, fluxes and rate coefficients were measured with radioisotopes and standard chemical techniques. The intracellular (vacuole) potential was measured with microcapillary electrodes and a high-impedance voltmeter.

2. Intracellular ion concentrations were estimated after correcting for 89% tissue water and 19% extracellular space. Intracellular concentrations of the major ions were (meq./kg. cell water): Na^+, 66; K^+, 680; Cl^-, 462. All of the

intracellular ions except I^- appeared to be completely exchangeable and were probably in solution in the large cell vacuole.

3. Nernst equilibrium potentials, calculated for each ion under conditions of approximate flux equilibrium, were (mv.): Na^+, +50; K^+, −105; Rb^+, −127; Cs^+, −85; Cl^-, −4; Br^-, −14; and I^-, +128. Comparison of these values with the measured vacuole potential of −81 mv. suggested active extrusion of Na^+ and active uptake of K^+, Rb^+, Cl^- and Br^-. Cesium was in approximate electrochemical equilibrium, and the status of I^- was uncertain due to some intracellular binding.

4. Cation fluxes were stimulated by light and, to a lesser extent, by exogenous substrate (glutamate). Previous exposure to light had a temporary stimulating effect on cation fluxes, provided oxygen was present. Anaerobiosis (nitrogen) depressed both influx and efflux of cations. Rate coefficients for the cation were in the order $Na^+ > K^+ > Rb^+, Cs^+$.

5. Anion fluxes were variably affected by light. Chloride uptake was promoted by light, Br^- uptake was slightly stimulated, and I^- uptake was depressed. Anion efflux, however, was consistently stimulated by light. Anaerobiosis inhibited the uptake of all anions. Rate coefficients for anion fluxes were similar to each other but were generally lower than the rate coefficients for cation fluxes.

6. With the exception of I^-, neither light nor anaerobiosis had much effect on the steady-state distribution of ions in *Gracilaria*. The intracellular I^- concentration was much higher in the dark than in the light. This was also found in several other seaweeds, and appeared to be due to an increased amount of bound I^- in non-photosynthesizing tissues.

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BEHAVIORAL AND INTEGUMENTARY CHANGES ASSOCIATED WITH INDUCED METAMORPHOSIS IN DIEMICTYLUS[1]

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After metamorphosis the newt, *Diemictylus viridescens,* passes into a terrestrial (eft) phase for two or three years, following which the animal migrates to water and assumes adult characteristics. This return to the natal environment has been termed a "second metamorphosis" by Wald (1960). Grant and Grant (1958), following the suggestions of Chadwick (1941), have shown that migration to water and associated skin changes from the granulated to smooth condition can be initiated in the hypophysectomized eft with 1.4 μg. of highly purified prolactin (Li). Similar responses have been produced in efts by Masur (1962) following pituitary autotransplantation, which suggests an inhibitory control exerted by the hypothalamus over the release of water-drive principles from the adenohypophysis. Waterman and Grant (1961) reported 48-hour thyroidal uptake of I^{131} to be 15.8% of original dose in efts as compared to 1.8% in adults. Prolactin-treated efts showed a shift towards adult values with lowered uptakes at 6.9%.

According to Etkin (1964) primary metamorphosis in urodeles can be compared with anuran metamorphic climax, as it also involves the growth of glands and pigment and the degeneration of caudal fins and external gills. The concept that thyroxin is associated with tail fin degeneration has recently been reinforced by Gross (1964), who found that collagenolytic activity of tail fin tissue from thyroxin-treated tadpoles increased significantly over that of controls. The process of tail resorption is complicated, involving a laying down of new collagen fibers in basement membranes and lysis of older fibers by collagenase released from epidermal cells. Gradual dissolution of basement membranes is the prime factor in tail regression. Kaltenbach (1953) has shown the effect of thyroxin in producing localized metamorphosis in *Rana pipiens* tadpoles, and Kaltenbach and Clark (1965) have demonstrated the direct effect of embedded pellets of thyroxin and thyroxin analogues on the skin of *Diemictylus viridescens.*

Activity of the pituitary-thyroid axis during primary metamorphosis is too well known to be reviewed here, but mention of some pituitary-hypothalamic relationships is essential. Transplantation of adenohypophysial primordia in *Rana pipiens* tadpoles reported by Etkin (1938), and Etkin and Lehrer (1960), and sectioning of the infundibular stalk in *Ambystoma* larvae by Etkin and Sussman (1961) indicate that some continuity between the hypothalamus and hypophysis is necessary to sustain TSH release during metamorphic climax. As larvae operated upon in this manner continue to grow, the possibility exists that amphibian prolactin released after removal of hypothalamic inhibition acts as a growth factor. Recently,

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the important paper of Berman *et al.* (1964) has substantiated the role of prolactin as a larval growth factor following intraperitoneal injection of highly purified prolactin and bovine STH into *Rana catesbeiana* tadpoles.

Other factors occurring during primary metamorphosis have not been studied in detail. For example, there is still considerable controversy over the relationship of thyroxin to metabolic rates; no increase in rate was reported during metamorphosis in *Rana grylio* by Lewis and Frieden (1959), while Barch (1953) found increased oxygen consumption in isolated skin from metamorphosing larvae and in skin from the area of thyroxin-pellet implants. Kaye (1961) showed an increase in thyroid activity during metamorphosis in *Rana pipiens* tadpoles in which 43% I^{131} uptake occurred at the end of the premetamorphic period.

Gorbman (1964) suggested that molting, an important characteristic of amphibian skin not restricted to periods of metamorphosis, should be considered as two separate processes, the first involving proliferation and cornification of the epidermis and the second sloughing of epidermal structures. This concept is validated by the fact that experimental procedures which inhibit sloughing do not necessarily affect proliferation of the epidermis. The production of local molts in the area of thyroxin-pellet implants in thyroidectomized animals by Clark and Kaltenbach (1961) and the extended series of experiments of Adams and her co-workers (Adams, 1932; Adams and Grierson, 1932; Adams and Richards, 1929; Adams *et al.*, 1932, etc.) would indicate that molting activity is conditioned by the direct action on the skin by thyroxin whose production is regulated by a pituitary-thyroid control mechanism. That the molting process may be more complex is evidenced by the work of Chadwick and Jackson (1948) and Grant and Grant (1958) on *Diemictylus* (*Triturus*) *viridescens*, where prolactin increased epidermal mitotic rates in normal animals and elicited irregular sloughing following hypophysectomy. Both the thyroid and adrenal cortex may activate slough preparation and sloughing to varying degrees in *Ambystoma* and *Bufo*, according to Jørgensen and Larsen (1960, 1961), who also consider that frequency of molting is maintained by levels rather than cycles of thyroid activity.

The present investigation was undertaken in order to explore further the control exerted by both thyroxin and prolactin on primary and secondary metamorphosis in *Diemictylus*, with special attention directed towards related epidermal changes.

MATERIALS AND METHODS

The efts and adults of *Diemictylus* used in these studies were obtained freshly caught from a supplier, while the larvae were collected locally during the summer. In the first series of experiments four groups of 20 adult animals received injections every other day according to the following protocol: group A, 0.1 ml. amphibian Ringer's; groups B and b, 5 μg. of L thyroxin sodium in 0.1 ml. of amphibian Ringer's until the end of four days when it was increased to 20 μg. per injection; group C, 100 μg. of ovine prolactin (NIH) in Ringer's solution. A fifth group of 20 animals, D, was placed in a terrestrial environment on moss (mostly *Polytrichum*). Each of groups A, B, b, and C was placed in a "choice" situation which consisted of plastic containers (6 × 10 in.) separated into equal areas of land and water by plastic partitions ¾ in. high. This allowed the animals to move freely from one area to another. Each container held five test animals and daily observations were made to determine the distribution of animals in either of the two environments. On the eleventh day of this experiment groups B and C were divided into three groups each: one which continued to receive the original treatment, one which received no further treatment, and one which received the reciprocal of its original injection (*i.e.*, thyroxin-treated animals received prolactin and *vice versa*). Half of group D was now offered a land-water choice situation and group A continued as controls. In another experiment each of 36 efts was injected with 100 μg. of prolactin in order to study skin changes associated with induced water migration.

Histological sections of skin cut at 7 μ were prepared from the mid-dorsal and lower jaw region of animals in each experimental group and stained with either Harris' hematoxylin-eosin or Masson's trichrome stain. Photomicrographs of slides were made with a Unitron MiC3-369 microscope.

In the second series of experiments larvae of *Diemictylus* were divided into pre-melanic and melanic groups, the latter representing the darkened phase occurring just prior to primary metamorphosis. Animals were maintained in plastic cups in which a stender dish covered with moist filter paper provided a land environment. Pre-melanic animals (0.12 gm. average weight) were divided into two groups; one received intraperitoneal injections of 0.01 ml. Ringer's solution twice a week by glass capillary needle and another 10 μg. prolactin at the same volume. Another group was placed in sterile pond water containing 1 μg./ml. thyroxin. Three groups of melanic stage animals received similar treatment. Periodic observations were made of metamorphic events on the staged scale of pre-melanic, melanic, gill loss, and land migration which terminated primary metamorphosis. These stages correspond to the phases of primary metamorphosis reported by Noble (1929) for *Diemictylus*.

Groups of adults which were shifted to a land environment were injected intraperitoneally with 1 microcurie of radiodide after ten days on land. Counts of thyroids were made at 24, 48, 72 and 96 hours on a scintillation counter, and uptakes expressed as a per cent of the injected dose of I^{131}. Uptakes of a control group of aquatic adults were taken at 24 and 72 hours. All experiments reported above were conducted at approximately 22 ± 1° C.

Other investigations were made to determine the direct effect of thyroxin and prolactin on newt skin in tissue culture. Adult newts were anesthetized in 0.1% MS222, immersed briefly in a $5 \times 10^{-4}\%$ aqueous merthiolate-Ringer's solution and placed in a sterile cabinet. Tissue sections 1 mm. square were excised from the mid-dorsal region of the animals under a mercury vapor lamp (GE #G8T5) and placed for an hour in deep-well slides containing the antibiotic salt solution of Fimian (1959). Three tissue fragments were placed in each of a number of Carrel D-50 flasks on a 1-ml. coagulum containing chick plasma (Difco), chick embryo extract (Difco EE-100) and antibiotic solution in a 3:1:5 ratio. Tissue fragments were then covered with 2 ml. of antibiotic solution (0.6 gm./liter of penicillin and streptomycin) which was changed every three days. Control tissues were incubated in saline solution while experimental tissues were treated with solutions of either 0.2 mg./ml. thyroxin or 1 mg./ml. prolactin (NIH and highly purified samples by Li). Tissue fragments were removed periodically for histological analysis.

RESULTS

Water-land migration in adults (Fig. 1)

Thyroxin-treated animals began to emerge from the water on the sixth day and by the eleventh all members of group b and 80% of group B were terrestrial. A large percentage of animals in group B which continued to receive thyroxin treat-

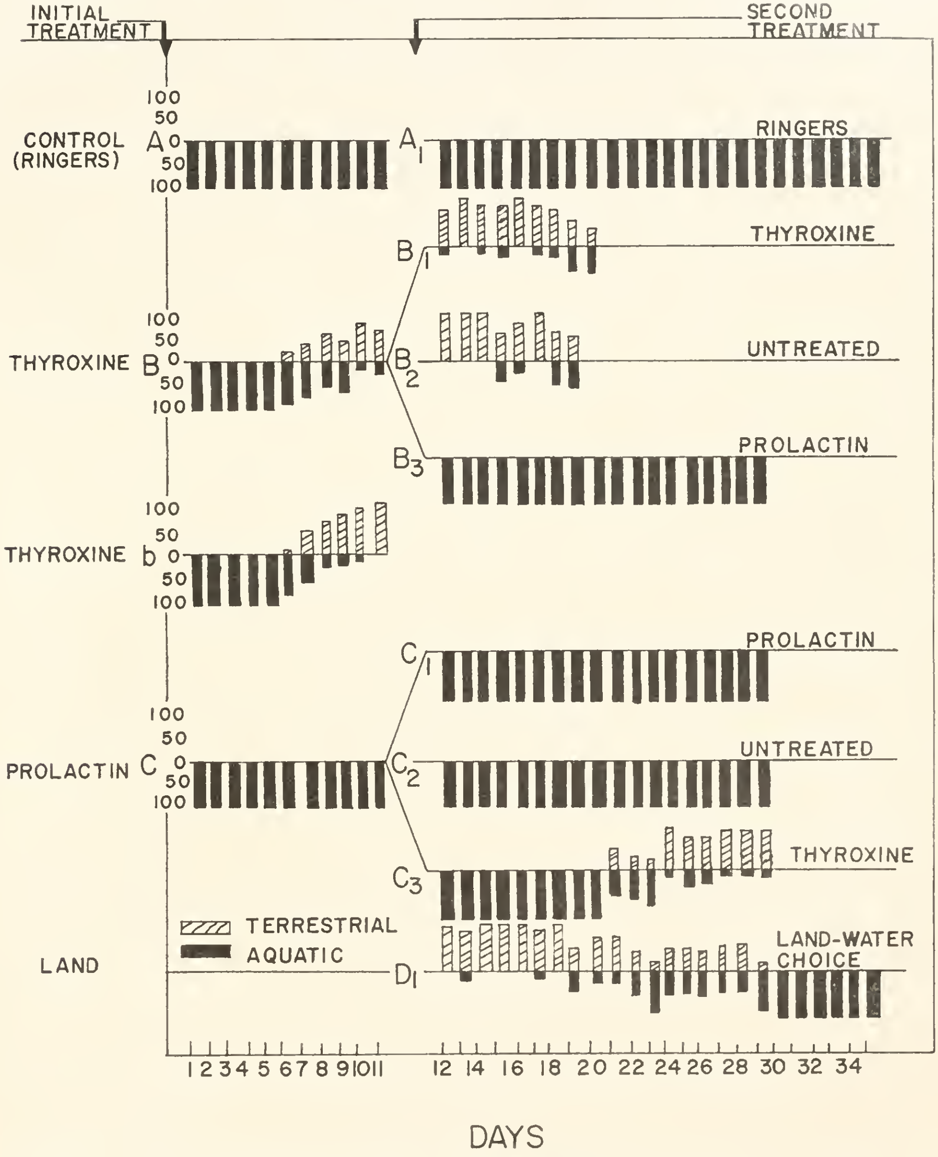


FIGURE 1. Per cent distribution of adults of *Diemictylus* in choice cages with aquatic or terrestrial environments, following treatment with thyroxin and prolactin.

ment or which were left untreated after the eleventh day continued on land although some returned to water towards the termination of the experiment. Group B animals which received prolactin on the twelfth day became aquatic within a day and remained so until termination of the experiment.

No members of the control group A or the prolactin-treated group C were ever observed on land during the entire course of the experiment. In those group C animals treated with thyroxin after cessation of prolactin injections a land-drive occurred approximately 10 days after the initiation of thyroxin injections, as compared to the six-day latent period for group B animals. Animals were recorded on land an average of 88% of the time during the last three days of the experiment which began 15 days after the first treatment with thyroxin.

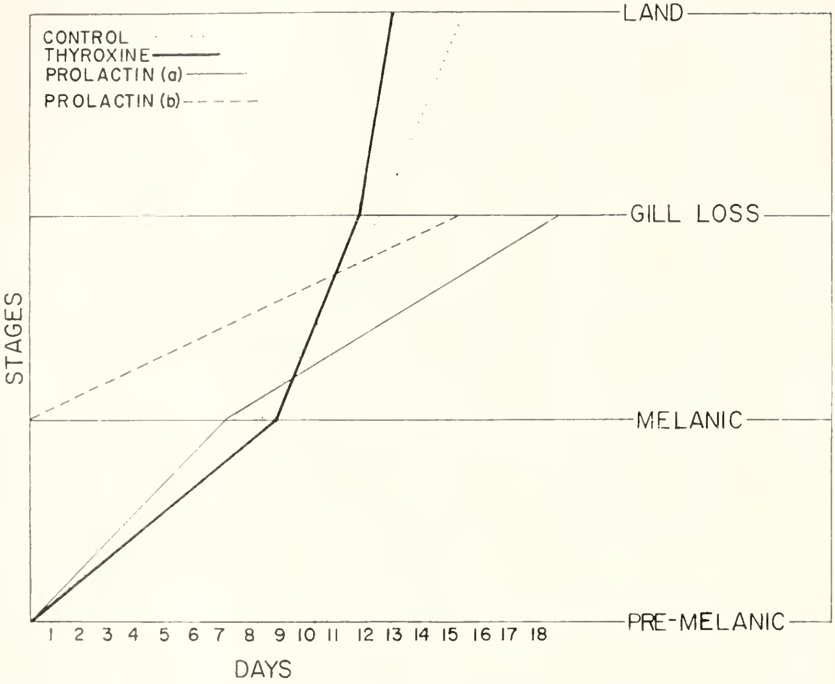


FIGURE 2. Average number of days for larvae of *Diemictylus* to pass through pre-melanic, melanic, and gill loss stages following injection of thyroxin or prolactin.

Group D animals which had been land-conditioned on moistened *Polytrichum* showed a gradual shift to water which was completed by the eleventh day when they were presented with a choice of environments.

Effects of thyroxin and prolactin on events during primary metamorphosis (Fig 2 and Table I)

The average time taken for thyroxin-treated larvae and controls to proceed from the pre-melanic stage through complete metamorphosis was 12.8 and 15.3 days, respectively. This time differential was largely accounted for by the accel-

Table I

Average time in hours from initial injection for larvae of Diemictylus to pass through stages of primary metamorphosis

Treatment	Control	Thyroxin	Prolactin-Group I	Prolactin-Group II
Number of animals	17	6	6	5
Pre-melanic to melanic	199.2 ± 15.6	208.8 ± 40.6	168 ± 36.4	
Melanic to gill loss	297.6 ± 18.3	273.6 ± 21.9	348.8 ± 19.3	364.8 ± 6.1
Gill loss to complete metamorphosis	367.2 ± 5.3	307.2 ± 6.0	—	—

erated rate of metamorphosis in thyroxin-treated animals as compared with controls from the time of gill reabsorption to their emergence on land which in the former occurred within a 24-hour period.

An event of major significance in these experiments was the failure of prolactin-treated larvae to complete metamorphosis despite the fact that their gills had been reabsorbed as much as two weeks before the termination of observations. In addition, they showed a lower rate of metamorphosis between melanic and gill loss stages, taking as long as 15 days to make the transition, as compared to an average of 72 hours for the controls and thyroxin group. The time taken for prolactin-treated animals to pass through the pre-melanic stage was slightly less than that

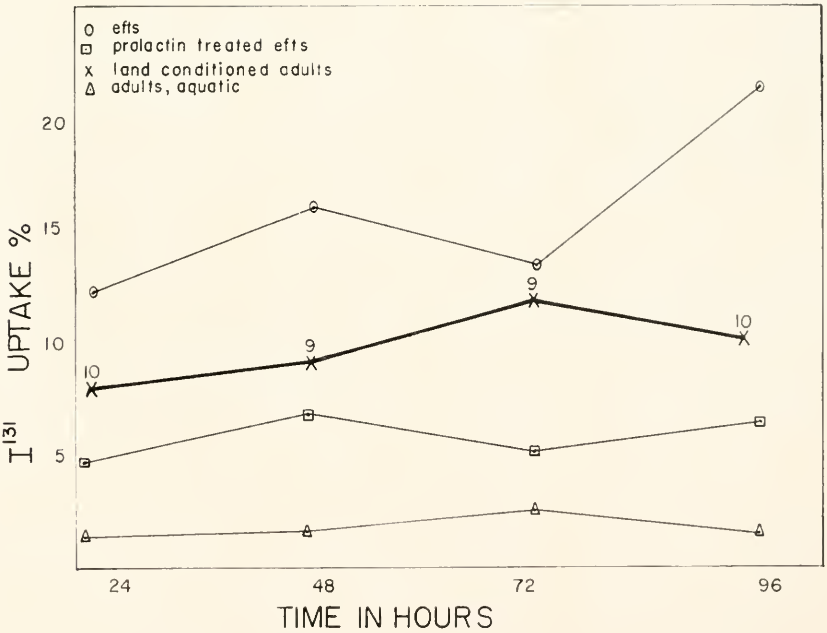
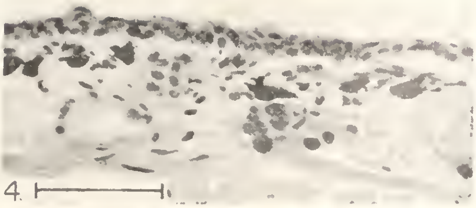


Figure 3. Per cent thyroid uptake of I^{131} by efts, prolactin-treated efts, normal adults and land-conditioned adults. The number of land-conditioned animals used in the determination at each point is indicated. Data on other phases are modified from Waterman and Grant (1961).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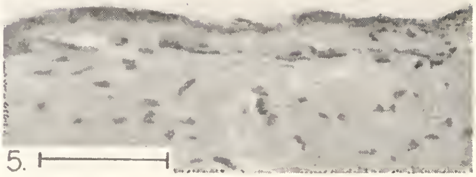
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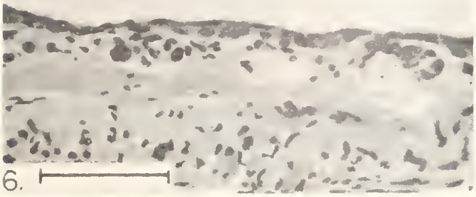
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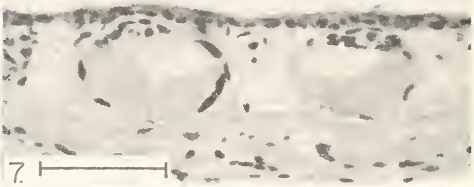
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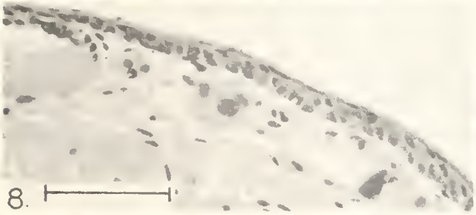
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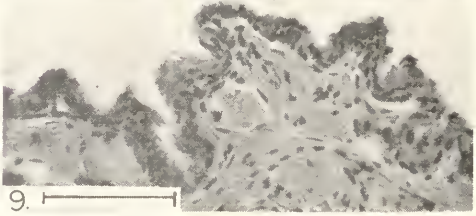
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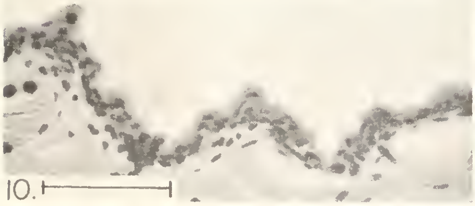
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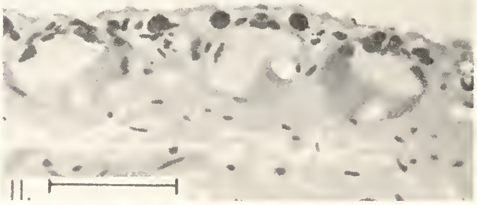
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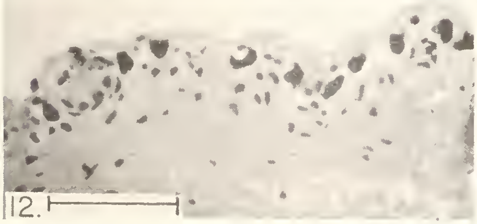
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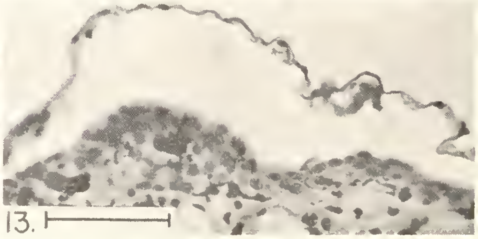
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FIGURE 4. A section of skin taken from the back of an adult newt. The scale is 50 μ. Hematoxyln and eosin.

FIGURE 5. Back skin of adult animal (Group B) following two-week treatment with thyroxin, showing reduction of epidermis and glands. The scale is 50 μ. Hematoxylin and eosin.

FIGURE 6. Neck skin of a newt five days after cessation of thyroxin treatment, showing some epidermal thickening and enlargement of glands. The scale is 50 μ. Hematoxylin and eosin.

FIGURE 7. Skin from the neck of an adult newt after 45 days of prolactin injections. The epidermis is extremely smooth and glandular elements are well developed. The scale is 50 μ. Hematoxylin and eosin.

for the other groups. However, as there was no precise way of determining the actual age of pre-melanic animals at the time of their first injection, slight variations at this level cannot be considered particularly significant. It is interesting to note that larvae which remained aquatic after gill loss made appropriate behavioral adjustments by coming to the surface to obtain air in the manner described by Spurway and Haldane (1954) for adult newts, despite the fact that a favorable surface-to-volume ratio for cutaneous respiration undoubtedly existed.

Thyroid activity of terrestrial adults (Fig. 3)

The following daily values, expressed in per cent uptake of I^{131} by thyroids of adult animals maintained on land for a 10-day period, were recorded: 8.1 ± 1.9%, 24 hours; 9.3 ± 3.2%, 48 hours; 12.07 ± 0.9%, 72 hours; 10.2 ± 2.8%, 96 hours. In Figure 3 these values are compared with those previously taken for efts, normal adults and prolactin-treated efts by Waterman and Grant (1961). In the present study controls showed uptakes of 1.6 ± 1.1% and 0.6 ± 0.51% at 24 and 72 hours, which compare closely with those for normal adults in previous studies.

Histological and tissue culture studies

Because the results of tissue culture studies were best observed at the cellular level they are discussed here for comparative purposes with results obtained from histological studies of eft and adult skin.

A skin section from a normal adult is shown in Figure 4. Animals from group B showed an increase in glandular and molting activity several days after treatment with thyroxin. In two weeks, however, when these animals had migrated to land, their glands were either absent or greatly reduced and their epidermis was composed of a thin layer of cells covered with several layers of *stratum corneum* (Fig. 5). Figure 6 shows a return to the normal adult condition in animals from group B_2 five days after cessation of thyroxin injections. Glandular elements are present once more and there has been considerable epidermal proliferation. Thyroxin-treated animals underwent complete molts several days after initial injections but failed to do so after their emergence on land.

In general, prolactin treatment had similar results in both adults and efts. In these animals there was a marked and sustained increase in the size of mucous glands, and their epidermis became compact and smooth with a thin cornified layer.

FIGURE 8. Back skin of an eft four weeks after a single injection of prolactin (100 μg). The epidermis is smooth and compact. The scale is 50 μ. Hematoxylin and eosin.

FIGURE 9. Normal eft skin, showing folding of epidermis and presence of cornified tubercles. The scale is 50 μ. Hematoxylin and eosin.

FIGURE 10. Skin from adult newt of Group D kept in a terrestrial environment for six weeks. The rough, granular epidermis clearly resembles that of the eft (Fig. 9). The scale is 50 μ. Hematoxylin and eosin.

FIGURE 11. Section of adult skin maintained in organ culture for two weeks. Epithelium is a single, squamous layer and dermal atrophy is beginning. The scale is 50 μ. Hematoxylin and eosin.

FIGURE 12. Thyroxin-treated tissue culture of adult skin is shown at two weeks. There is a general disorganization of the dermis, melanophore migration, and a definite epidermis is lacking. The scale is 50 μ. Hematoxylin and eosin.

FIGURE 13. Prolactin-treated skin culture at two weeks showed a compact, proliferating epidermis which underwent occasional molts. The scale is 50 μ. Hematoxylin and eosin.

Figure 7 shows the skin of an adult of group C 40 to 45 days after initiation of treatment with prolactin and Figure 8 that of an eft 27 days after a single injection of 100 μg. prolactin. In the case of the eft there are major skin alterations involving the loss of skin folds and tubercles as compared to the granular skin of the normal eft (Fig. 9). It is interesting to note that in adults from group C there was a reversal of prolactin-induced changes by subsequent thyroxin injections, with the appearance of a more corrugated skin and reduced skin glands over a 15-day period. However, there is indication that previous prolactin treatment increased the latent period of thyroxin activity, if comparisons are made of this group and previously untreated group B animals which received thyroxin injections for the same period of 15 days.

Group D adults placed on land showed little skin change at first, but after several days there was a progressive increase in the size of their skin glands, followed by a thickening and folding of the epidermis. Eventually the skin became virtually indistinguishable from the normal eft condition, as can be seen by comparing skin from adults kept on land for 47 days (Fig. 10) with normal eft skin shown in Figure 9. Land-conditioned animals which returned to water assumed normal adult skin structure within 10 days. The molting activity of group D adults was similar to that of efts as the *stratum corneum* was constantly shed and replaced in patches rather than being sloughed as a unit.

It is clear that the skin of the adult animal can adjust readily to shifts in environment. Prolactin produced skin changes in the adult which paralleled those occurring in normal efts at the time of second metamorphosis. Thyroxin at the levels used in the present investigation, however, could only recapitulate some primary metamorphic changes in the adult, for although thyroid-treated animals responded by migrating to land, an eft-like epidermis never formed.

Adult skin sections in tissue culture were maintained as controls for a period of at least two weeks. At the end of five days there was considerable epidermal degeneration and many of the larger glands had been emptied of secretion. At 15 days (Fig. 11) dermal structure and glands were still reasonably well organized but tissue was covered with a single, loosely organized layer of squamous epithelium. In thyroxin-treated cultures glandular atrophy and degeneration of the epidermis occurred within a period of several days, and at two weeks (Fig. 12) the skin had lost most of its structural organization as compared with controls. Melanophores had migrated to the surface and a definitive epidermis was completely lacking. In contrast, prolactin-treated cultures were well maintained over a period of several weeks with little degeneration or distortion of cellular and tissue elements. The epidermis remained compact and underwent several molts during the course of the experiment. These were observed floating in wash solutions above the tissue fragments, as seen in Figure 13. No molts were observed in either the control or thyroxin cultures.

DISCUSSION

The investigations described above indicate that profound changes occur in the behavior and skin structure of *Diemictylus* during the course of endocrine-induced primary and secondary metamorphosis. Thyroxin induces a land-drive in adults and acceleration of metamorphosis in larvae, while prolactin treatment initiates water-drive in efts and adults and inhibits metamorphosis of larvae. However, the causes of these events and such associated changes as skin morphology, molting and thyroid activity, etc., are complex.

It is generally accepted that the thyroid is involved with primary metamorphosis and molting mechanisms in urodeles. Clark and Kaltenbach (1961) and others have suggested that molting is directly influenced by thyroxin. The present studies indicate that, in addition, environment may play a significant role in determining both molting activity and skin conditions.

Thyroxin-injected adults showed a land-drive, but neither whole animals nor tissue cultures treated with thyroxin produced the heavy granular eft skin associated with the terrestrial habitat. Thyroxin had a deleterious effect on the integrity of the epidermis, which suggests either inhibition of growth of the *stratum germinativum*, or destruction of basement membranes, or both. Although some initial stimulation of molting did occur, it did not resemble that of the eft. Normal larvae in primary metamorphosis and adults kept on land, however, did develop eft-like skins. A possible interpretation is that assumption of the terrestrial environment itself stimulates the endogenous release of factors, including thyroxin, which control and maintain the eft condition after the organism metamorphoses from the larval state under the primary influence of thyroxin. The failure of thyroxin to maintain a viable skin in the present experiments is supported by the collagenolytic effect of thyroxin on basement membranes reported by Gross (1964).

Thyroid activity of terrestrially maintained adults, as indicated by per cent uptake of I^{131}, approaches the high values shown for efts by Waterman and Grant (1961) in contrast to the low uptakes reported for aquatic adults and prolactin-treated efts. Even if the limitations of the technique are considered, it is safe to conclude that increased thyroid activity is associated with events during and following primary metamorphosis and that this activity is decreased during secondary metamorphosis. Despite considerable controversy (Lewis and Frieden, 1959; Barch, 1953) about the effect of thyroxin on metabolic processes in amphibians, the above results suggest that increased thyroid activity may be necessary to meet the greater metabolic demands of the terrestrial organism.

The immediate effects of prolactin treatment on newts appear to be considerably more direct than those following thyroxin administration. With reasonable certainty prolactin can be considered to be the water-drive principle in *Diemictylus*. It appears to maintain the integrity of the characteristic type of epidermis formed during normal second metamorphosis in the intact animal and in tissue culture. For example, larvae treated with prolactin not only failed to undergo primary metamorphosis but assumed the characteristic skin structure of aquatic adults. The effects of prolactin on tissue growth and maintenance noted above are supported by the work of Chadwick and Jackson (1948), Berman *et al.* (1964), and by Niwelinski (1958), who found that prolactin stimulated differentiation in forelimb regenerates of *Triturus alpestris*. The relationship of prolactin activity to molting, however, remains obscure, although Grant and Grant (1958) reported partial molting after prolactin injections in hypophysectomized efts. Some stimulation of molting by prolactin is consistent with the view that epidermal proliferation is a distinct factor of the molting mechanism (Chadwick and Jackson, 1948).

The evidence assembled to date indicates that prolactin and thyroxin have certain antithetical properties with respect to their influence on metamorphic events in urodeles. Thyroid activity is directed by the hypophysial release of TSH under neurosecretory stimulation from the hypothalamus (Etkin, 1938, 1964; Etkin and Lehrer, 1960; Etkin and Sussman, 1961). Hypothalamic activity in turn may inhibit prolactin secretion (Masur, 1962). While major shifts in the levels of either thyroxin or prolactin may help produce the adjustments to environment associated with metamorphosis, undoubtedly a delicate balance exists between those factors which help to maintain the normal integument of both the aquatic and terrestrial forms during and after metamorphic events. This is apparently true in the case of anuran molting in which environmental and adrenocortical factors may be involved as well (Jørgensen and Larsen, 1960, 1961; Stefano and Donoso, 1964). Prolactin in general appears to have a more complete influence on conditioning independent physiological adjustment to environment than thyroxin, for whereas adults and even gill-bearing larvae (Noble, 1929) can be kept in a moist, terrestrial environment, efts can only be maintained in water after prolactin administration.

The possible evolution of prolactin and thyroxin activity paralleling the course of vertebrate evolution is suggested by these studies. The thyroid hormone and changes in response to its activity undoubtedly conditioned the assumption of terrestrial modes of life in Amphibia, whereas prolactin-like principles may have been initially involved with osmoregulatory forces operating upon the integument of organisms in fresh-water habitats and later with the water-drive responses associated with second metamorphosis and annual spawning activity. Therefore, one would expect a balanced thyroxin-prolactin system effecting terrestrial and aquatic adaptation to be most clearly defined in the more generalized urodele amphibians which indeed appears to be the case. Nicoll and Bern (1964) failed to demonstrate the presence of prolactin in organ cultures of teleost and chondrichthyian pituitary tissue with pigeon-crop assay. Grant and Pickford (1959) and Grant (1961), however, obtained positive water-drive response in efts treated with teleost, shark and skate pituitary brei. This suggests the possibility of either a range in the structure and activity of prolactin-like substances throughout the vertebrates, or progressive evolution in vertebrates of a parent "prolactin molecular entity," as suggested by Nicoll and Bern and Berman *et al.* (1964). One of the earliest activities of prolactin (or prolactin-like principles) may have been involved with maintaining osmoregulatory homeostasis in fresh-water organisms through its effect on integumentary systems. This suggestion is supported by the fact that mammalian prolactin affects fresh-water survival in *Fundulus* and in *Xiphophorus,* as shown by Pickford and Phillips (1959) and Schreibman and Kallman (1964) and the increased water-drive activity in pituitaries of *Bufo americana* and *B. fowleri* taken from ponds during the spawning season (Grant, unpublished). This homeostatic activity which was present in the earliest fresh-water vertebrates may be directly affected by the "water-drive" principle which is present throughout the vertebrate series. On the other hand, the prolactin growth factor which is associated with pigeon-crop-stimulating activities, as indicated by Berman *et al.* (1964), may have originated more recently at the time of tetrapod evolution in the Devonian. The taxic response shown by prolactin-treated efts in locating water (Grant, 1964) indicates that the water-drive principle has a direct effect on behavior *per se* in addition to its action on the integument. Such activity would, of course, have been essential to the first terrestrial vertebrates.

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SUMMARY

1. After larval metamorphosis the eft (terrestrial) phase of *Diemictylus viridescens* can be induced to undergo second metamorphosis to the aquatic, adult phase with mammalian prolactin. In the present investigations adults receiving thyroxin (10 to 20 μg. every other day) showed a partial reversal to second metamorphosis by migrating to land. However, the thin, cornified epidermis did not approximate the granular and compact eft-like epidermis seen in adults which were maintained without treatment in a terrestrial environment.

2. Ovine prolactin (NIH) produced water migration in efts and inhibited primary metamorphosis in larvae, while maintaining the smooth, compact epidermis characteristic of the normal adult.

3. Reaction of adult skin fragments in tissue culture receiving either 0.2 mg./ml. thyroxin or 1 mg./ml. prolactin paralleled the above results: after several days thyroxin-treated skin had lost most of its structural organization as compared with controls while prolactin cultures were well maintained, with a compact epidermis which even molted on several occasions.

4. Iodine-131 uptake of land-maintained adults showed values (12.07 ± 0.9%) which approached those for efts, as compared to the low values for normal adults and prolactin treated efts.

5. The evolution of a prolactin-thyroxin axis during the course of vertebrate evolution is suggested. Thyroxin may have affected adaptation to terrestrial environments and prolactin adaptation to the fresh-water habitat. Although the early activity of prolactin may be associated with the water-drive principle, it is understood that changes in tissue response or in the structure of prolactin have greatly extended the range of action for prolactin (prolactin-like principles) in higher vertebrates.

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DEVELOPMENTAL STAGES OF THE BOB-WHITE QUAIL EMBRYO (COLINUS VIRGINIANUS)

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Since the well-known work by Hamburger and Hamilton (1951) on normal chick embyros appeared, a number of workers have "staged" embryos of various species of birds. They clearly established morphological and structural characteristics as more reliable criteria than chronological age in the staging of chick embryos. Rempel and Eastlick (1957) showed constant differences between embryos of the White Silkie bantam and standard varieties of the domestic chicken. Koeke (1958) described the embryonic stages of the domestic duck. Mun and Kosin (1960) correlated the development of broad-breasted bronze turkey embryos with the chick. Although few significant differences were recorded, they showed that the turkey embryo has a distinctive structure, the "snood" or "leader," which serves as an important criterion for older stages. Hanson (1954) applied candling and special photographic techniques in determining the age of mallard, wood duck and bob-white quail embryos. His primary purpose was to determine the age of embryos at the nest site.

In the present paper, normal stages of chick embryo development (Hamburger and Hamilton, 1951) were used as the basis for establishing the stages of development for the quail. It is known that the quail embryo takes approximately 23 days to hatch, as compared with 21 days in the chick. Thus, the major objective of this study was to correlate the developmental stages of quail and chick embryos, and to identify the periods of incubation when the quail embryo "lags" behind in development.

Since all photographs, descriptions and measurements are based on material fixed in FAA (5 ml. formalin, 90 ml. 70% ethyl alcohol, 5 ml. glacial acetic acid), minor distortions may have occurred. Freshly laid fertile eggs were obtained from the State Game Farm, Mount Vernon, Illinois. They were incubated in a Farm Master incubator at a temperature of 99.5° F. dry bulb and about 89.0° F. wet bulb. During the course of this study several hundred embryos were examined from the unincubated stage until hatching.

No constant differences were observed between quail and chick embryos through stage 6 (25 hours) (Hamburger and Hamilton, 1951) and reference to these stages will be omitted. Between stages 7 and 14, which are based primarily on the number of pairs of visible somites, the quail embryo lags in development so that by stage 14 (22 somites) it is 12–16 hours older than the chick. The gradual lag in developmental rate continues so that by stage 24 the quail embryo is 24 hours behind the chick embryo in morphological development. Flexion and rotation of the quail embryo are slightly more advanced but amnion formation is slightly lagging up to stage 24; otherwise, the differentiation of conspicuous structures shows an even "slowing down." This comparison of development rates is continued in Table I.

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Descriptions will be presented only of the succeeding stages for which the criteria used for chick staging and other notable features will be applied to the quail. We have not staged 4–8-day embryos at half-days as did Hamburger and Hamilton (1951); consequently, several stages of chick development were grouped to correspond with morphological development in the quail. The morphological details of half-day stages for the chick will serve as excellent guides if one considers the one-day lag in incubation time of the quail.

TABLE I

A comparison of incubation times and stages of development for quail and chick embryos

Quail		Chick	
Days of incubation	Stage of development	Days of incubation	Stage of development
5	24	4–4½	24,25
6	25	4½–5	25,26
7	26	5½–6	28,29
8	27	6½–7	30,31
9	28	7½–8	32,33
10	29	8–9	34,35
11	30	10	36
12	31	11	37
13	32	13	39
14	33	13	39
15	34	14	40
16	35	15	41
17	36	16	42
18	37	17	43
19	38	18	44
20	39	19–20	45
21–22	40	20–21	46*
23	41*		

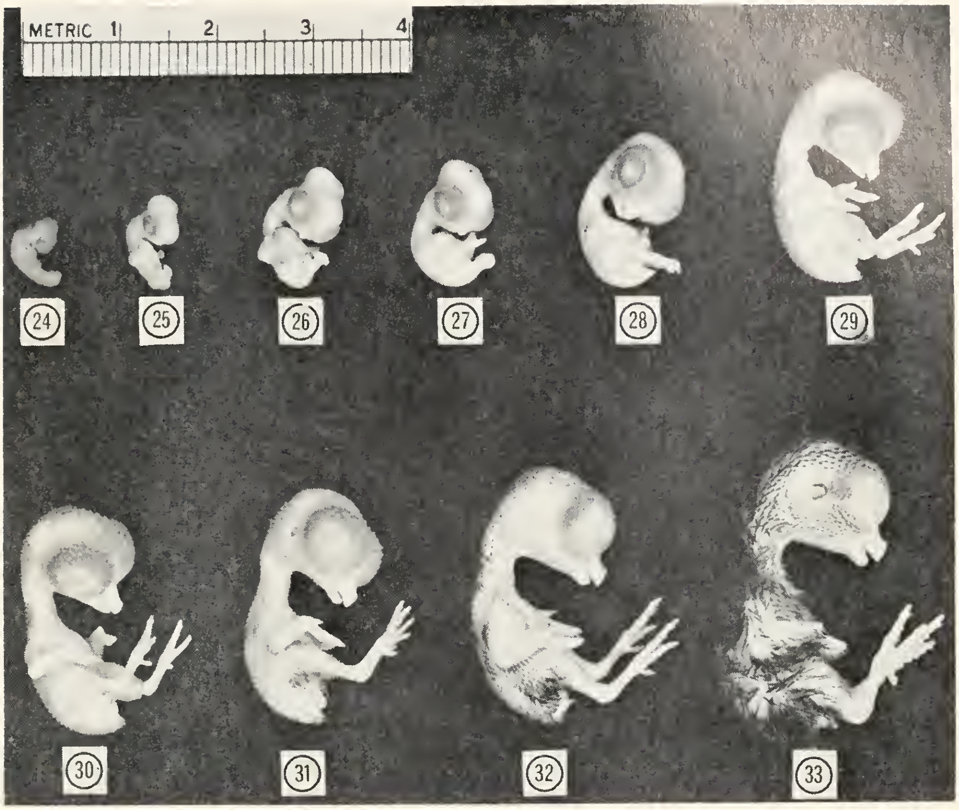
* Newly-hatched chick.

We are recording the average measurements of the beak and third toe for the quail as did Hamburger and Hamilton (1951) for the chick, and will also record the total length of the foot as Rempel and Eastlick (1957) did for the bantam. This measurement is taken from the outside of the tarsal joint to the terminus of the claw of the third toe.

The descriptions that follow should be used in conjunction with the illustrations, which are numbered according to stages.

Stage 24 (*ca.* 5 days) (stage 24, chick)

1. Limbs: Both wing and leg-buds approximately the same size; and distinctly longer than wide. Digital plate in wing not yet demarcated. Toe-plate in leg-bud barely distinct.



All numbers in the following plates refer to the corresponding stage numbers in the text. Consult the description of each stage for a more complete explanation of the figures.

PLATE 1

Stages 24–33

2. Visceral arches: Almost identical to comparable stage in the chick. Second arch overlaps third arch and part of the fourth arch which is indistinct. Third and fourth visceral clefts visible but inconspicuous.
3. Olfactory pits visible.

Stage 25 (*ca.* 6 days) (stages 25 and 26, chick)

1. Limbs: Longer than previous stage. Contour of digital plate slightly rounded or angular. Three opaque bars, representing the toes, are visible, with the more translucent intermediate tissue constituting the "groove." Tip of third toe not yet pointed.
2. Visceral arches: Maxillary process fused with wall of nasal groove (notch visible at point of fusion). The distal tip of the second arch, known as the "collar," completely overlaps visceral arches III and IV. The third and fourth visceral clefts are no longer visible.
3. Beak: Faintly recognizable in profile.

Stage 26 (*ca.* 7 days) (stages 28 and 29, chick)

1. Limbs: Wing bent in elbow joint. Second digit distinctly longer than others. Shallow grooves visible between all the digits. Leg bent slightly in knee joint. Grooves present between toes. Webs indicated between toes. Distal contours of webs are slightly concave lines. Rudiment of fifth toe never visible topographically in the quail.
2. Visceral arches: Mandibular process lengthened and approaches the beak. External auditory meatus distinct; elongated to semi-lunar in shape. Neck has lengthened conspicuously between "collar" and mandible. "Collar" still very conspicuous but flattening.
3. Beak: Visible from all angles. No egg tooth.
4. Scleral papillae: Not visible.

By Stage 28, or approximately the eighth day of incubation, the morphological characteristics of the quail are delayed so an 8-day quail embryo corresponds to a 6½- to 7-day chick embryo.

Stage 27 (*ca.* 8 days) (stages 30 and 31, chick)

1. Limbs: Webbing still clearly visible between digits. Contour of webs concave. All four toes more conspicuous. Webs between toes thinner and contour definitely concave.
2. Visceral arches: Mandible elongated and approaching tip of beak. "Collar" visible but inconspicuous.
3. Feather-germs: None visible. Position of humeral tract distinct from dorsal view.
4. Scleral papillae: At least one but usually two on the dorsal side, and two or more on the opposite side. Papillae appear on the dorsal surface first.
5. Nictitating membrane: Visible, but not conspicuous at anterior surface of eye.
6. Egg-tooth: Distinct, protrudes as a sharp pointed whitish dot from upper mandible only.

Stage 28 (*ca.* 9 days) (stages 32 and 33, chick)

1. Limbs: Webs between digits and toes are thin and their contours are concave. Web on radial margin of arm and first digit discernible. Individual digits and toes conspicuous.
2. Visceral arches: Anterior tip of mandible has almost reached the beak. "Collar" has disappeared and cleft associated with collar barely visible. Neck has lengthened.
3. Feather-germs: One distinct row runs on each side of the brain (in the groove between the eye and the brain) from the anterior edge of the eye to the base of the head. Approximately twelve rows on dorsal surface at level of legs. One row distinct on each side of tail. At least one row visible on humeral tract and four rows on femoral tract. Dorsal apterium visible. Several rows on ventral side of neck faintly visible. Feather tract beneath wings distinct.
4. Scleral papillae: Twelve to fourteen, forming a complete circle.
5. Nictitating membrane: Extends over half-way between outer rim of eyelid and scleral papillae.

Stage 29 (*ca.* 10 days) (stages 34 and 35, chick)

1. Limbs: Webs between digits and toes inconspicuous. Phalanges of toes are distinct. Second digit lags behind three and four. Third toe has grown longer than others. Digit four distinct.
2. Visceral arches: Overall lengthening of beak apparent.
3. Feather-germs: At least three rows comprise capital tract (on inner side of each eye). Flight feathers (posterior edge of wing) extend to distal tip. Feather-germs on thigh visible. Ventral surface, feather-germs extend from sternal area to each side of umbilical cord. Feathers on sides of tail, femoral tract and in lumbosacral area have black pigmentation.
4. Nictitating membrane: Approaches or partially covers the outer scleral papillae. Eyelids begin to grow over the eye-ball. The circumference of the eye-ball is becoming elliptical.

Stage 30 (*ca.* 11 days) (stage 36, chick)

1. Limbs: Length of third toe from its tip to the middle of its metatarsal joint = 4.4 ± 0.3 mm. Primordia of claws are visible on termini of the toes. Protuberance of digit 4 still present. Length of foot = 7 ± 0.3 mm.
2. Visceral arches: "Labial groove" visible on upper jaw in profile. Nostril has formed to a slit. Length of beak from anterior angle of nostril to tip of bill = 1.9 mm. Channel of external auditory meatus distinct.
3. Feather-germs: Feathers are visible on the caudal tract, on femoral tract and in lumbosacral region. Feather-germs extend about one-third of the way down lateral side of leg and almost to the phalanges on lateral surface of wing. Feather-germs faintly visible along ventral surface of external auditory meatus.
4. Eyelids: Nictitating membrane covers antero-most scleral papillae and approaches cornea. Lower lid has grown upward and approaches cornea. Circumference of lids is a narrowing ellipse with its ventral edge flattened.

Stage 31 (*ca.* 12 days) (stage 37, chick)

1. Limbs: Claws of toes only slightly flattened laterally and curved ventrally. Pads on plantar surface of foot visible. Transverse ridges along the superior surface of the metatarsus are barely visible as first indication of scales. Inferior surface of metatarsus and surfaces of phalanges smooth. Protuberance (on posterior side of digit 2) is missing. Length of third toe = 5.2 ± 0.3 mm. Length of foot = 9 ± 0.3 mm.
2. Visceral arches: Labial groove present. Length of beak from anterior angle of nostril to tip of bill = 1.5 mm.
3. Feather-germs: Elongated into long, tapered black cones on spinal tract, femoral tract and lateral to tail. Cones just visible on humoral tract. Spinal tract cones extend from base of skull to tail. Feather-germs visible around external auditory meatus. Primordia not visible on circumference of eyelids. Sternal tracts contain 7–8 prominent rows when counted at anterior end of sternum.
4. Eyelids: Nictitating membrane has reached anterior edge of cornea. Upper lid has not reached dorsal edge of cornea. Lower lid covers one-third of

cornea. Circumference of lids now bounds a much-narrowed elliptical surface.

The presence or absence of the labial groove will be recorded but it does not serve as a good criterion for staging in the quail.

Stage 32 (*ca.* 13 days) (stage 39, chick)

1. Limbs: Primordia of scales marked off on superior surface of legs; appear to be ready to overlap. Primordia (transverse ridges) on remaining surfaces of leg visible. Pads on plantar surface are conspicuous. Length of third toe = 6.1 ± 0.3 mm. Length of foot = 12 ± 0.3 mm.
2. Visceral arches: Length of beak from anterior angle of nostril to tip of bill = 1.7 mm.
3. Feather-germs: Coverts of web conical. External auditory meatus surrounded by feather-germs and short brownish-black cones conspicuous around anterior half. Lateral edge of sternal tract lined with brownish cones, medial portion with white cones visible. Both eyelids lined by several rows of feather-germs. Brownish cones showing on head and upper leg.
4. Eyelids: Nictitating membrane covers anterior surface of cornea. Lower eyelid covers one-half to two-thirds of cornea.

Stage 33 (*ca.* 14 days) (stage 39, chick)

1. Limbs: Scales overlapping on superior surface of leg. Primordia of scales are marked off on inferior surface. Small conical projection from tip of second digit. Lateral and medial surface of leg appears smooth or covered with fine feather papillae. Foot pad covered with papillae; pads of phalanges are smooth or papillae barely discernible on proximal pads. Length of third toe = 7.2 ± 0.3 mm. Length of foot = 14 ± 0.3 mm.
2. Visceral arches: The channel of the external auditory meatus is distinct and long slender feathers project over its ventral edge. Length of beak from anterior angle of nostril to tip of bill = 1.85 mm.
3. Feather-germs: Coverts of web of wing are very long tapering cones. Remaining portion of wing primarily covered with "white" feather-germs which are elongated. Border of external auditory meatus three-fourth of the way surrounded with elongating brown cones.
4. Eyelids: Opening between lids much reduced. Black pigment visible on edge of lower eyelid and barely visible on edge of upper eyelid.
5. Raised area and U-shaped slit at site of future uropygeal gland.

Stage 34 (*ca.* 15 days) (stage 40, chick)

1. Limbs: Length of third toe = 18.2 ± 0.3 mm. Scales overlapping on superior surface of leg and beginning to overlap on inferior surface. Main plantar pads and lateral surfaces of phalanges covered with large papillae. Area between pads and surfaces of web also covered with papillae. Claw forming on second digit. Length of foot = 15 ± 0.3 mm.
2. Visceral arches: Periderm intact. Length of beak from anterior edge of nostril to tip of bill = 1.98 mm. The main channel of the external auditory meatus is visible but completely surrounded by long black feather-germs.
3. Feather-germs: Entire dorsal and lateral surface of embryo covered by "colored" elongated "feather-cones" except on eyelid and radial aspect of

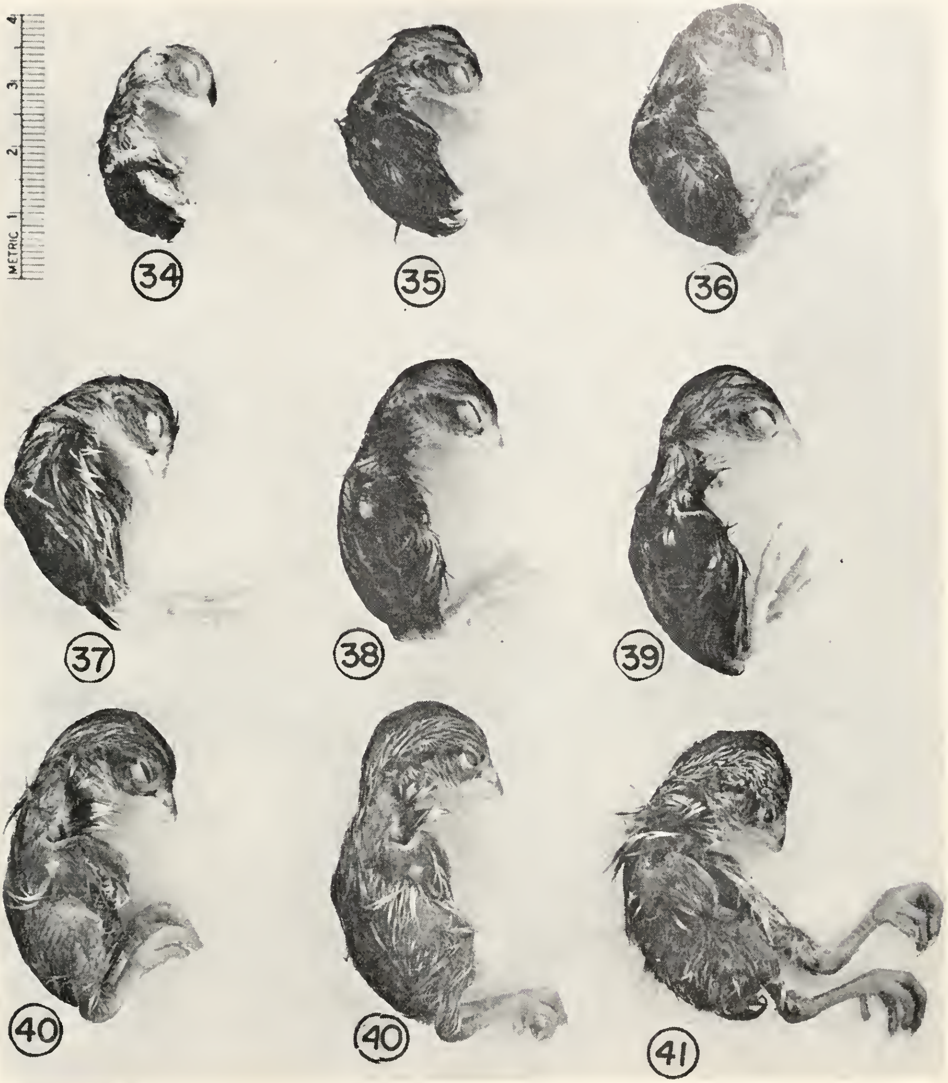


PLATE 2

Stages 34–41

wing, where feather-germs are short and white. Ventral surface covered by long white cones. Medial surface of wing and web covered by white feather-cones.

4. Uropygeal gland appears as a raised area, covered with circular pattern of feathers smaller than those of the back, with a crescent-shaped slit along its posterior border.

Stage 35 (*ca.* 16 days) (stage 41, chick)

1. Beak: Length from anterior edge of nostril to tip of upper bill = 2.2 mm. Periderm intact. Dorsal outlines of border of external auditory meatus still visible.
2. Limbs: Length of third toe = 10.0 ± 0.3 mm. Scales overlapping on superior and inferior surfaces of leg. Entire plantar surface of phalanges covered with large papillae. Blunt cone projects from tip of second digit. Length of foot = 17 ± 0.3 mm.
3. Feather-germs: All "colored" except under side of throat, part of sternal tract and lower eyelid.
4. Uropygeal gland: A feathered fleshy tab covering crescent-shaped slit under its posterior border.

Stage 36 (*ca.* 17 days) (stage 42, chick)

1. Beak: Length from anterior edge of nostril to tip of upper bill = 2.27 mm. Periderm beginning to break at proximal end of beak.
2. Limbs: Length = 11.0 ± 0.5 mm. "Claw" forming on second digit. Length of foot = 20 ± 0.5 mm.
3. Feather-tracts: Cover lower eyelid.
4. Uropygeal gland is a fleshy projection with feathers on tip. Begins to resemble adult condition.

Stage 37 (*ca.* 18 days) (stage 43, chick)

1. Beak: Length from anterior angle of nostril to tip of upper bill = 2.50 mm. Periderm on tip of upper mandible peeling off or already missing. Dorsal surface of upper mandible brown. Sides of upper mandible and lower mandible white.
2. Limbs: Length of third toe = 11.7 ± 0.8 mm. Length of foot = 22 ± 0.8 mm. Feet becoming brownish or tan in color, particularly at upper portion. Claw on second digit is slightly curved toward body.

Stage 38 (*ca.* 19 days) (stage 44, chick)

1. Beak: Length from anterior angle of nostril to tip of upper bill = 2.66 ± 0.3 mm. (Variation dependent upon amount of periderm peeled from beak.) Periderm gone from most of beak but still covers egg tooth. Beak becoming shiny, brown or tan colored. External auditory meatus well hidden by feathers.
2. Limbs: Length of third toe = 13.0 ± 0.8 mm. Superior surface of foot tan in color. Length of foot = 23.7 ± 0.8 mm. Claw on second digit curves sharply toward body.

Stage 39 (*ca.* 20 days) (stage 45, chick)

1. Beak: Length from anterior angle of nostril to tip of bill = 2.9 mm. Periderm about one-half gone. Beak is more pointed than it was previously. Upper bill dark brown with white streak.
2. Limbs: Length of third toe = 13.5 ± 0.8 mm. Upper surface of leg light brown and tips of scales blackish. Length of foot = 25 ± 0.8 mm.
3. Turbinate septum or bone forming in nostril. No change in claw of second digit or uropygeal gland.

Stage 40 (*ca.* 21–22 days)

1. Beak: Length from anterior angle of nostril to tip of bill = 3.1 mm. Length is less diagnostic because periderm is sloughed off. Egg tooth is a sharp whitish prong on upper mandible.
2. Limbs: Length of third toe = 15.7 ± 0.8 mm. Scales on legs becoming uniformly brown. Growth of second digit small compared to that of third. Second digit and claw are becoming inconspicuous. Length of foot = 28.5 ± 0.8 mm.
3. Extra-embryonic membranes: Yolk-sac is at least partially enclosed in body cavity. Turbinate bone or septum visible in nostril on both sides of beak.

Stage 41 (*ca.* 23 days) (stage 46, chick)

Hatching or newly-hatched chick.

1. Beak: Egg tooth present or absent on one of three embryos. Length of beak from anterior angle of nostril to tip of bill = 3.2 mm.
2. Limbs: Length of third toe = 16.5 ± 0.8 mm. Length of foot = 29 ± 1 mm. Claw on second digit unchanged, entire digit inconspicuous due to accelerated growth of carpometacarpus.
3. Extra-embryonic membranes: Yolk-sac is entirely or half-enclosed in body-cavity. Opening still visible.

We wish to express our gratitude to the Department of Zoology and to the Cooperative Wildlife Research Laboratory, Southern Illinois University, for laboratory space and equipment. We also wish to acknowledge the aid of the Illinois State Game Farm, Mount Vernon, Illinois, for supplying us with freshly-laid fertilized bob-white quail eggs.

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STUDIES ON SPERMATHECAL FILLING IN AEDES AEGYPTI (LINNAEUS). II. EXPERIMENTAL[1]

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Our knowledge of the physiology of sperm transport in the vertebrates is very extensive (see, *e.g.*, Parkes, 1960, and Bishop, 1961), but relatively little experimental information is available for insects. Most of what is known about sperm transport in insects has been well reviewed by Wigglesworth (1953) and Hinton (1963). It is generally believed that the female is largely or entirely responsible for translocating sperm to her storage organs in many Hemiptera (Davey, 1958), Coleoptera (Blunck, 1912), Lepidoptera (Hewer, 1934; Omura, 1938; Callahan and Cascia, 1963), and Hymenoptera (Ruttner, 1956; Snodgrass, 1956). In some Hemiptera, the sperm are said to be injected by the male directly into the spermathecae (Ludwig, 1926; Bonhag and Wick, 1953). In certain insects chemotactic substances within the female are claimed to be important in sperm transport (*e.g.*, Weidner, 1934). On the other hand, in *Drosophila*, it has been suggested that the sperm are largely or entirely responsible for their transport within the female (Nonidez, 1920). In *Anopheles* mosquitoes, Giglioli (1963) stated (p. 166) that ". . . ejaculation appears to be the direct passage of spermatozoa through the phallotreme into the spermathecal duct . . . ," and he suggested (p. 160) that sperm transport could be ". . . best explained by the existence of a partial vacuum in the spermatheca during insemination; this being achieved by peristaltic contractions of the . . . [spermathecal] duct." Descriptions of spermathecal filling in *Aedes aegypti* are given by Burcham (1957a), Schwartz (1961), Spielman (1964), and by Jones and Wheeler (1965).

In the present study, an attempt was made to identify the major factors involved in the filling of the spermathecae after insemination of the Bangkok strain of the yellow fever mosquito. Specifically, it was our aim to determine whether spermatozoa locomote within a passive female reproductive tract, whether some action of the female is solely responsible for their translocation, or whether both motility of the sperm and the female's activity are required to fill the spermathecae. We have by no means resolved the question, but this paper sets forth the problems that must be solved before we shall have a very clear understanding of sperm transport in aedine mosquitoes.

METHODS

The mosquitoes were reared as previously described (Jones and Wheeler, 1965). In order to manipulate the mosquitoes, they were first anesthetized with

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nitrogen. Two techniques were used to study sperm transport. The first one involved rubbing the terminalium of the male against that of the female at an angle of about 90° under a dissecting stereo-microscope at a magnification of 13 ×. The dorsal surface of the thorax of each mosquito had previously been glued to the head of a separate pin and the manipulations were made using Wheeler's apparatus (1962). Basically, this is the technique of McDaniel and Horsfall (1957) and will be referred to hereafter as *forced-copulation* or *forced-mating*. The second method involved injection of the bursae of virgin females with a variety of materials, using a fine glass micropipette and the assembly described by Wheeler and Jones (1963). The great majority of the artificial inseminations were made using *Drosophila* Ringer (Ephrussi and Beadle, 1936) buffered to pH 6.8. The microscope cold-stage used in some of the experiments was that described by Wheeler (1964). For other details see the text.

RESULTS

1. *Effect of the female's position on spermathecal filling*

Immediately following free mating, *Aedes* females perch themselves so that their abdominal tip faces down. To determine whether shifting the normal posture of the freshly inseminated female would affect spermathecal filling, immediately after forced-copulation, females were oriented into the following positions: abdominal tip facing (a) up, (b) down, (c) to the side, or (d) the female was turned upside down. Four to 9 females were used for each position. In each case two to three thecae filled with sperm, and the results in no way differed from those of females which had been force-copulated and released into a cage. Thus, spermathecal filling is not affected by the position of the female.

2. *Effect of certain surgical operations on spermathecal filling*

In one experiment the heads of 6 virgins (4–7 days old) were crushed with forceps before presenting them to unmated males for forced-copulation. Only three of the females force-copulated. Of these three, one was not inseminated. One had ejaculate on the outside of her genital lips and apparently some within her vagina, but no sperm were seen in the bursa or thecae. However, the third female had been properly inseminated: the bursa was fully distended with ejaculate and two of her thecae contained numerous sperm 64 minutes after coitus. Five control females all readily force-copulated and transferred sperm to the thecae.

The experiment was repeated using 17-day-old mosquitoes. Of the 6 females used, one did not copulate with any of three potent males. One female did not accept the first male but did accept the second; this copulation lasted 350 seconds, but the female was not inseminated. The other four females all force-copulated with the first male presented, and received seminal material, but only three of them had few to many sperm in two thecae.

Five 3-to-5-day-old virgin females were decapitated and all 5 of them force-copulated with previously unmated males (they copulated for 6 to 43 seconds, with a mean of 17.6 seconds).

Ten 15-day-old virgin females were decapitated and all 10 force-copulated with 15-day-old males. Most copulated for an abnormally long time (21 to 242 seconds, with a mean of 91.2), but only 6 were inseminated and only four had few to many sperm in two thecae.

These experiments indicate that crushing the head or decapitation of the female prior to mating may lead to erratic findings with forced-copulation.

Heads of four females were crushed during the act of forced-copulation (*i.e.*, as soon as the male had made full genital contact), and the females were dissected in 5 to 12 minutes. All four females had been inseminated and all had sperm in two to three thecae. Heads of 5 females were crushed immediately after withdrawal of the male, and the females were dissected in 16 to 17 minutes. One of the females had not been inseminated, but the other four females had sperm in two to three thecae. Thus, damaging the head of the female during or immediately after forced-coitus in no way interferes with spermathecal filling.

Five females were force-copulated for 15 seconds and within 30 seconds, or less, their terminalia cut off and placed in an open drop of saline on a glass slide. Four minutes later the thecae were dissected. Four of the 5 females had been inseminated. Of the 15 thecae examined, three had many sperm, two had few to many sperm, three had very few sperm, and 7 had no detectable sperm. The results did not obviously differ from controls. Thus, spermathecal filling is controlled by forces within the terminalium alone.

On four occasions, where the intact bursa, vagina and spermathecal complex had been isolated into a drop of saline and covered with a layer of immersion oil within less than 30 seconds after mating, we observed the empty thecae suddenly fill with many spermatozoa. In one case the sperm did not fully enter the thecae but vibrated within the base of the large theca like a sheaf of delicate spears. In another case, many sperm shot suddenly into the median theca and quickly began to spin around within it. In two other cases, two thecae completely filled with sperm. In all four cases, the thecae filled within the first 45 seconds of the observations and very rapidly stopped. Unfortunately, the spermathecal ducts were not clearly visible in any of these preparations, but the bursal orifice and vestibule were visible in two cases and the sperm were exceedingly active in these regions. Thus, spermathecal filling can occur *in vitro* and the forces responsible for it are located in a highly specific portion of the female reproductive tract.

Seven virgin females which had fed on blood three days previously were force-copulated for 15 seconds and immediately thereafter were decapitated to induce rapid egg deposition. Five of these females began to lay eggs. The ovipositing females were dissected within 5 to 10 minutes. All five of them had two thecae filled with numerous sperm. This is not proof, however, that the thecae filled with sperm during egg deposition because the females were dissected after the sperm are known to be able to reach the thecae and the sperm could have easily ascended during the intervals between egg depositions. The eggs did not hatch. This technique is currently being used to study the important question of sperm capacitance.

3. *Effects of exposure to nitrogen, ether, carbon dioxide and cyclopropane on spermathecal filling*

Although nitrogen quickly immobilizes *A. aegypti,* they recover quite rapidly when placed in air. Five females were force-copulated for 15 seconds and placed in a steady stream of nitrogen. After being hosed with nitrogen for four minutes, they were dissected in about 45 seconds. All 5 females had sperm in two thecae. Of the 15 thecae examined one had many sperm, 5 had few to many sperm, four had very few sperm, and 5 had no sperm. Identical results were obtained with 5 controls kept in air for four minutes.

Three females were placed in ether vapors for 15 to 30 seconds and afterwards quickly force-mated for 10 to 21 seconds. The females recovered in less than one minute and were dissected after 5 to 10 minutes. Two of the females had been inseminated, and both of these had active sperm in their distended bursae and few to many sperm in one to two thecae. Four females were force-mated for about 17 seconds and then quickly etherized for one to two minutes. They were allowed to stand in air for 5 minutes before dissection. All of the females had few to many sperm in two thecae. Ten females were force-mated for 10 to 15 seconds, allowed to stand in air to 30 to 90 seconds, then placed in strong ether vapors until dead and were dissected. All of the females had their bursae distended with ejaculate, and all had few to many sperm in one to two thecae. Five females were force-copulated for 10 to 15 seconds, allowed to stand in air for one minute, then placed in ether vapors for one to five minutes, and then dissected. All females had few to many sperm in one to two thecae. In three of the females, the thecal sperm were active.

Five females were forced-mated for 15 seconds and placed in a stream of carbon dioxide for four minutes and dissected within one minute. Four out of the 5 females had few to many sperm in one to two thecae; the sperm were very intensely active in both the bursae and thecae. Of the 15 thecae examined, one had many sperm, three had few to many sperm, 5 had very few sperm, and 6 thecae had no sperm. The results did not obviously differ from controls, except that carbon dioxide seems to increase sperm activity.

Five females were force-copulated for 15 seconds, and in one to three seconds were placed in a stream of cyclopropane where they were quickly immobilized. They were hosed for 5 minutes and dissected in 28 to 51 seconds. Four of the females had been inseminated, and each of them had sperm in two thecae. Of the 15 thecae studied, two had numerous sperm, three had few to many sperm, three had very few sperm, and 7 thecae had no sperm. The results did not differ from controls.

Thus, a variety of gaseous anesthetics which externally immobilize the female do not necessarily affect spermathecal filling.

4. *Effects of chilling on sperm and spermathecal filling*

Ten females were force-copulated and within 5 seconds were completely submerged in an ice-water bath at about 7° C. for 10 to 20 minutes. The females were removed and dissected in ice-cold saline within approximately 30 seconds. One of the females had not been inseminated. Nine of the females had distended bursae with very active sperm. Of these 9 females, four had no sperm in the thecae. But 5 out of the 9 females had sperm in two thecae. Three females were force-mated and placed in an ice bath at 4° C. for 10 to 30 minutes. One of the females had not been inseminated. The other two females had the bursa with ejaculate containing active sperm, and in both of these cases a few sperm had reached the thecae. Five virgins were forced-copulated for 15 seconds, and in 8 to 10 seconds they were totally submerged in an ice-water bath at 1–2° C. for 30 minutes. They were dissected at room temperature within 32 to 41 seconds. Four of the females had sperm in two thecae, and one female had sperm in all three thecae. Of the 15 thecae examined, three contained numerous sperm, three contained many sperm, three contained few to many sperm, two contained only a few sperm, and four thecae had no sperm. Since it was possible that sperm could have started to reach the thecae during the seconds it required to dissect them, another experiment was made in which the females were dissected into a drop of cold saline on a special microscope cold-stage held at 1–5° C. Four females were force-copulated for 10 seconds, and in about 5 to 10 seconds they were submerged in an ice-water bath at 1–2° C. for 18 to 67 minutes. One of these females had a greatly distended bursa with numerous, dense, circular clusters of inactive sperm. The bursal wall was vacuolated and swollen, but the ejaculate was not vacuolated. No sperm were in the thecae of this female. In the second female the bursa was fully distended, and sperm were active at the bursal orifice but only a few active sperm were visible in the large theca and no sperm were in the lateral thecae; the ejaculate was not vacuolated. In the third female, the bursa was distended with non-vacuolated ejaculate, and a few sperm were active at the bursal orifice, but only a few sperm were present in two thecae. The fourth female was removed from the ice bath after 67 minutes, held at room temperature (27° C.) for about one minute, and then dissected. Many active sperm were present in two thecae. The ejaculate did not vacuolate during the 10-minute observation period.

To determine whether certain organs of the female would remain active at low temperatures, the hind gut and reproductive system were dissected into a drop of cold saline on the special microscope cold-stage held at 4° C. In one case, the hind gut and reproductive system did not contract during the 10-minute observation period. When the temperature was allowed to rise to about 7° C., the hind gut very slowly contracted; but the ovaries and lateral oviducts did not contract. In another case, held at 3 to 4° C., the hind gut and lateral oviducts very slowly contracted, but the ovaries were inactive. In a third case, the vagina and lateral oviducts did not contract at 1° to 4° C.

To determine whether sperm would remain active at low temperatures, the male reproductive system was isolated in an open drop of saline on a cold-stage held at 5° to 6° C. The sperm remained quite active in the posterior chamber of both testes for at least one hour and in the sperm ducts for about 30 minutes, but the sperm in the seminal vesicles were inactive. Sperm released into the saline remained actively undulating for about 6 minutes; those sperm within the vicinity of the ruptured seminal vesicles, however, remained active for about 30 minutes. At 1° to 2° C. the activity of the sperm seemed definitely greatly reduced or abolished.

These experiments indicate that spermathecal filling can occur at 4° to 7° C. at which temperatures the contractions of the female reproductive system are greatly reduced or abolished. At these same temperatures, however, the sperm remain quite active. At 1° to 2° C. spermathecal filling tended to be greatly reduced, the ejaculate in the bursa did not vacuolate, and the sperm were mostly inactive. That the sperm of *Aedes* can remain active for some time at 5° to 6° C. is in marked contrast to the sperm of *Periplaneta* which are mostly inactive at 10° C. (Richards, 1963). The sperm of the honey bee are reported to survive two hours "contact with ice" (Bishop, 1920).

5. *Effect of killing the female just prior to forced copulation on spermathecal filling*

Before being force-copulated, females were killed by overexposure to cyanide vapors (three cases), carbon dioxide (two cases), ether vapors (14 cases), or cold temperatures (7 cases). Of the 26 females used, two were unacceptable to a series of previously unmated and highly potent males. Of the 24 remaining females, 7 did not receive ejaculate. Males ejaculated on the outside of the genitalia of 8 (47.6%) of the 17 dead females. However, males deposited ejaculate in the bursae of 9 of the dead females. The sperm were inactive in the bursae of two of these. However, in 7 cases, the bursae of the dead females contained active spermatozoa. No sperm were seen in the thecae of these dead females at 10, 20, or 30 minutes after copulation. In most of the cases studied, the females were not severely dehydrated and the genital tract was generally not conspicuously blocked or otherwise distorted. However, overexposure to carbon dioxide can greatly distort the female's genital system (Spielman, personal communication) in such a way as to make sperm migration impossible.

6. *Distribution of dyes after injection into the bursa*

Using the apparatus of Wheeler and Jones (1963), various dyes in saline, with and without certain additives, were injected into the bursae of 114 virgins and the females dissected at varying intervals to determine the location of the dye. The data in Table I are based only on injected mosquitoes which appeared essentially normal.

As shown in Table I, (1) certain dyes injected only into the bursae of virgins are capable of being transported into the common oviduct, as far as the ampullae, and into one, two or all three thecae; (2) dyes may be detectable within the thecae without being visible within the thecal ducts; (3) the occasional presence of dyes in the periductal glands and in the basal glands of the spermathecae indicates that some of the dyes may be absorbed from the bursa into the hemolymph; (4) the addition of a variety of organic substances to the dye solutions did not seem to enhance the degree or extent of dye transport. In these studies, dyes were never found in the female's accessory gland or its duct. In all of the cases of bursal injection with dye, the bursal wall did not swell or develop vacuoles.

Some of the data in Table I need amplification. Four virgins whose bursae had been injected with congo red were force-copulated 16 to 21 minutes later, and the females dissected after standing for 20 to 24 minutes. The first female was not inseminated. The second mosquito had sperm in two thecae, but no dye was seen in any of her thecae. In the third case, sperm were in all three thecae but dye was questionably present only in the large median theca. In the fourth female, sperm were in all three thecae but dye was detectable in only two. No dye was seen in any of the spermathecal ducts.

The bursae of 5 virgins injected with congo red saline were force-copulated 20 hours later and dissected after 25 to 30 minutes. In the first female, sperm were in two thecae, but dye was detectable only in the bursa and common oviduct. In the second mosquito, the large theca was bright red, but no sperm were found in any of her thecae; very few sperm were visible in the bursa. In the third case, two thecae had sperm, but dye was clearly present in all three thecae; her common oviduct also had dye in it. Two of the mosquitoes had not been inseminated; and in both of them, dye was present in the large median theca only. Dye in those thecae which did not contain sperm probably was transported to them shortly after injection into the bursae.

TABLE I

Location of dyes within the female genital tract of Aedes aegypti after injection into the bursa. SD = spermathecal ducts; PGD = periductal glands

Solution injected	No. ♀♀	Time dissected	Location of dye (no. cases)						
			Bursa	Oviduct	SD	PDG	Spermathecae		
							Median	Lateral	
								1	2
Methylene blue	1	15 min.	1	1	0	0	0	0	0
Amaranth red	5	32-109 min.	5	3	0	0	2	2	1
	12	24 hrs.	12	3	1	7	2	0	0
+sonofied milk	10	24 hrs.	10	2	4	4	0	1?	0
+ glycogen	15	19–24 hrs.	15	1	3	12	3;1?	0	0
+ 10^{-4}% noradrenalin	8	20 hrs.	8	0	2	4	0	0	0
+ 2% egg albumin	3	20 hrs.	3	0	0	0	0	0	0
+ extract ♂ reprod. system	3	19 hrs.	3	0	2	2	1	2	2
+ extract 20 ♂ acc. glands	5	22 hrs.	5	1	3	3	3	1	0
Sudan red in Shillaber's oil	1	10 min.	1	0	0	0	0	0	0
Congo red	7	20–59 min.	7	4	2	1	1	0	0
	5	32–109 min.	5	3	0	0	2	2	1
	10	115-150 min.	10	5	1	0	2	2	1
	5	20 hrs.	5	3	0	0*	1;1?	0	0
+ fructose	5	96–100 min.	5	1	0	0	1	0	0
+ force cop. after 16–21 minutes	4	20–24 min.	4**	0	0	0	2;1?	1;1?	0
+ force cop. after 20 hrs.	5	25–30 min.	5**	3	0	0^{ϕ}	4†	1	0
Cage cop. 3 hrs; then inject bursa with congo red	10	90–117 min.	10	5	1	0	1‡	1‡	1‡

* Orange in basal glands of spermathecae.
** Ejaculate in three females' bursae.
ϕ Dye in basal glands of three females.
† Dye in one theca of both uninseminated females.
‡ Dye in all three thecae of the one uninseminated female.

Two to three hours after free-copulation, the bursae of 10 females were injected with congo red saline and the mosquitoes were dissected in 90 to 117 minutes. Nine of them had been inseminated. Dye was present in all bursae and in the common oviduct of 5. Dye was visible in all three thecae of the one uninseminated female and *not* visible in any of the inseminated females' thecae. This suggests that dye transport to the thecae ceases after the thecae fill with sperm.

How the dyes become distributed to the thecae is not clear. Whatever the explanation, it seems evident that a mechanism exists within the virgin female reproductive system which could direct sperm into the thecae, and this mechanism stops when the thecae fill with sperm.

7. *Distribution of various sperm mixtures after injection into the bursa*

The bursae of 94 females were successfully injected with many to very numerous usually highly motile sperm under a variety of experimental conditions. The females were not roughly handled before, during or after artificial insemination, and although some injury may have occurred, it is considered minimal for the cases reported here. The data may be summarized in the following statements. (1) Pure undiluted seminal vesicle sperm did not reach the thecae within 84 minutes (one case). (2) In one out of 6 cases, after injection of seminal vesicle sperm in saline into the bursa, only a very few sperm reached one theca in 10 to 150 minutes. (3) A very few sperm reached the large theca in two out of 8 cases within 19 to 24 hours after injection of the bursa with seminal vesicle sperm in amaranth red saline. (4) When noradrenalin ($10^{-4}\%$) and extracts of either the testes or of the whole male reproductive system were added to seminal vesicle sperm in saline, no sperm reached the thecae (10 cases). (5) In two out of 10 cases injected with seminal vesicle sperm in saline plus male accessory gland exudate, very few sperm reached only the large theca. When male accessory glands were ruptured in open saline drops, the exudate rapidly gelled, but when the glands were ruptured in saline under a layer of immersion oil, it was possible to withdraw exudate in suitable amounts.[3] (6) When seminal vesicle sperm were freshly collected into (a) fresh human seminal fluid (two cases), (b) sonofied sweet milk (four cases), (c) emulsified Shillaber's immersion oil (three cases), (d) testicular extract (one case), (e) fresh bull seminal fluid (one case), (f) fresh bursal ejaculate (three cases), and then injected into the bursae of virgin females, no sperm reached the thecae. (7) When fresh seminal vesicle sperm had been injected into the bursae and the females then quickly force-copulated with a sexually depleted male (*i.e.,* with a repetitively force-mated specimen) for one to 25 seconds, in only one out of 6 cases did a very few sperm reach the large theca. (8) Even when fresh ejaculate containing active sperm was quickly removed from the bursa of a freshly force-copulated donor female and this quickly injected into a recipient virgin bursa, sperm did not reach the thecae (three cases). (9) When seminal vesicle sperm were added to fresh ejaculates taken from a donor bursa and this injected into a virgin bursa and these recipient females then quickly force-copulated with a potent male for one to two seconds (*i.e.,* before the male could ejaculate), in three out of 5 cases only a few sperm were found in one to two thecae. (10) When seminal vesicle sperm were collected into a small drop of saline which was covered with a layer of immersion oil prior to injection into the virgin bursa, better results were obtained in one exceptional series of animals in one experiment. Thus, in 7 out of 17 cases in which fresh seminal vesicle sperm were collected in saline under a layer of oil, few to many sperm reached the large theca. (11) Testicular sperm collected in saline under oil did not reach the thecae (three cases). (12) Attempts to inject spermathecal sperm (4 to 9 thecae were needed for a single injection) were not satisfactory (two cases). (13) In one experiment fresh ejaculate was quickly removed from the bursa of a donor female into a small drop of saline and covered with a layer of immersion oil. In two out of three cases, many sperm reached one to two thecae. (14) Mammalian sperm from fresh human (two cases) and fresh bull ejaculates (four cases) placed in the bursae were very numerous, highly active, and small enough to pass into the spermathecal ducts, yet none left the bursa and none reached the thecae. In several cases, the alien sperm heads were oriented away from the bursal orifice.

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[3] Burcham (1957a, 1957b) apparently found no difficulty in micropipetting male accessory gland exudate from open saline drops, but our micropipettes consistently clogged.

Thus, while sperm withdrawn from the seminal vesicles or from a donor bursa and artificially injected into a virgin bursa are capable of reaching the thecae in about 25% of the cases, the extent and degree of thecal filling are very low and far inferior to that following either forced-mating or free-mating. Attempts to increase the extent of filling with artificially injected sperm were generally unsuccessful.

In many of the cases where sperm did not reach the thecae, the bursa was greatly distended with the injected material; and the sperm within the bursa were often very numerous, highly motile, oriented, and exhibited violent activity near the bursal orifice. In several cases, the sperm injected within the bursa locomoted violently around within the sac.

8. *Results of artificial insemination*

A small amount of information was collected on the results of artificial insemination of *A. aegypti*. Ten females were injected with fresh bursal ejaculate collected under oil, then released into a cage and the females given sugar water and offered a blood meal. Ten days after being injected, 6 females were dead and one was missing. The three remaining females laid 50 eggs, none of which hatched. When the females were dissected, there were no sperm in their thecae.

Thirteen 7-day-old virgins were artificially inseminated with fresh seminal vesicle sperm collected in saline under oil. The donating males were 21 days old and had not been mated. Three females were dissected in 10 to 20 minutes, and all three of them had few to many sperm in two thecae. The other 10 females were released into a cage. The next day, one female was dead. The remaining 9 females were offered a blood meal, which 5 of them took. Three to four days thereafter, a batch of 18 eggs was found. All of these eggs hatched and subsequently developed into fourth stage larvae. Inadvertently, these larvae were killed due to gross overfeeding. Fifteen days after being artificially inseminated, four females were dissected for study. Two of them had sperm in the thecae; one female had not been inseminated, and one female had inactive sperm in her bursa and no sperm in her thecae. The 5 remaining females were offered a second blood meal, and a batch of

8 eggs was subsequently found, all of which hatched and developed into active adult males and females.

Discussion

To account fully for spermathecal filling in the *Aedes* mosquito, one must explain how many spermatozoa leave a non-muscular sac, make a sharp U-turn, ascend highly convoluted but non-contracting ducts, and quickly enter fluid-filled spherical reservoirs which have no muscles.

There are several conceivable ways in which the female might play some role in sperm transport. First, the bursal wall might secrete a substance into the ejaculate which would be necessary for or useful to transport. There is no doubt that the bursal wall appears secretory after insemination, but this secretion appears to occur after sperm transport has already begun. Second, the female accessory gland might very briefly secrete a substance that would attract the sperm into the vestibule for only a short time. The female accessory gland is certainly well-situated for such a possibility. It is difficult to believe that the female accessory gland produces a chemically attractive substance, since seminal vesicle sperm fail to congregate about the gland or its orifice in *in vitro* preparations. Furthermore, there are no obvious histological differences in the secretory appearance of the female accessory gland before, during or after sperm transport. Presumably the gland is continuously secretory. Third, the fluid in the spermathecae or their ducts might have some chemically attractive substance occurring in a steep gradient. It seems improbable that the spermathecae have such a chemically attractive material because the sperm fail to congregate around the ducts in *in vitro* preparations and they do not specifically move towards crushed spermathecae in fresh whole-mounts. Fourth, the female might materially aid sperm transport by creating a current within the vagina. A current within this region could be produced by the brief rapid contractions of the transverse muscles of the spermathecal eminence as observed by Leahy (1962). Although such contractions might be elicited as a result of stimulation from the aedeagus during the act of coitus, we have not consistently seen such contractions immediately following forced-coitus in fresh dissections. An anteriorly-directed current is produced in the female genital tract by the vigorous rhythmic contractions of the lateral oviducts and ovaries (Curtin and Jones, 1961). This current might be regulated by closing or opening of the vaginal valves and/or the gonopore. Conceivably, an anteriorly-directed current within the vagina would help draw sperm out of the bursa into the upper vagina. Contractions of the muscles of the spermathecal eminence might then direct them into the vestibule itself. But, if vaginal currents do exist, two observations suggest they may not be crucial to spermathecal filling. In four cases, the spermathecae were observed to fill *in vitro* when no contractions of the vaginal area were visible and where the common oviducts had been mostly torn away. Contractions of the female genital tract are greatly reduced or abolished at 4° to 7° C. and yet some spermathecal filling can occur. The current produced by the contractions of the lateral oviducts is probably not involved in initially drawing sperm out of the bursa; otherwise sperm should quickly appear in the lower vagina and common oviduct and this occurs only about one hour after spermathecal filling is completed (Spielman, 1964; Jones and Wheeler, 1965). Fifth, the female might suddenly absorb fluid from one or more thecae into the hemolymph and this would produce a highly specific current. It is difficult to believe, however, that this could occur at 4° to 7° C. The entrance of spermatozoa into the already fluid-filled spermathecae must lead to some displacement of fluid in these organs. While each of the five possibilities referred to might be useful to sperm transport, we have no positive evidence that any one of them is involved in the filling of the spermathecae.

There are some obvious ways in which the activity of the spermatazoa might play an active role in spermathecal filling. First, the headpiece of these cells becomes highly oriented to certain interfaces and is kept in close contact with surfaces (Jones and Wheeler, 1965). Second, the spermatozoa are capable of explosive locomotion and violent spinning whirls, and these movements, together with the thigmotactic head, could account for the sharp U-turn from the bursa into the vestibule. These properties would not, however, explain why the sperm never attempt to enter the open orifice to the accessory gland duct. The speed with which sperm locomote could more than account for their ability to fill the thecae in less than 5 minutes; indeed, the striking thing is that they do not begin to enter the spermathecal ducts as soon as one would expect of such rapidly moving cells.

Artificially injected, active, oriented sperm within the bursa generally are not capable of reaching the thecae by themselves. Even in the normally mated female active sperm stop being transported to the thecae in 5 minutes or less, even though the bursal orifice is wide open and the vestibule is not closed by the ventral tuft (Spielman, 1964; Jones and Wheeler, 1965). That is, something obviously normally inhibits transport very shortly after normal insemination. Whether the female is partially responsible for this is not clear. Gelation and vacuolation of the ejaculate within the bursa cannot be entirely responsible because some sperm can reach the thecae in the absence of vacuolation (as in the cold experiments).

Whatever the explanation of our general failure to achieve good spermathecal filling following artificial insemination, it is certainly not due to failure to distend the bursa fully with numerous highly active spermatozoa capable of rapid locomotion on being released. Neither is it due to the lack of stimulation of the vagina by the aedeagus. Perhaps it is simply a matter of finding the right medium for the sperm but the fact that sperm in fresh ejaculates from donor females do not transfer makes even this seem unlikely.

Before spermathecal filling in *Aedes aegypti* can be understood we shall need to find out (1) what factors are involved in initiating the process once the bursa is inseminated (that is, why do the sperm "wait" before they start to enter the vestibule?), and (2) what factors are responsible for terminating the process after the thecae fill (that is, why do active sperm at the bursal orifice stop entering the vestibule when there is no mechanical blockade and when the third theca is generally never fully filled with sperm?).

A few comparisons may be made between sperm transport in *Aedes* and other insects. According to Nonidez (1920), the sperm of *Drosophila* are deposited initially in the "uterus," and those near the female's accessory gland ducts suddenly become active and swim into the tubular ventral receptacle, which fills in two to five minutes. In *Aedes,* there is no indication that sperm are activated by the female's accessory gland. Although Nonidez stated that after the ventral receptacle fills, the spermathecae then fill with sperm. DeVries (1964) reported sperm in the "storage organs" of *Drosophila* in less than one minute after coitus. Certain mutants of *Drosophila* which lack both accessory glands (parovaria) and spermathecae can produce viable offspring two to three days after copulation because the tubular receptacle alone can briefly serve as a storage organ (Anderson, 1945). Studies in this laboratory indicate that the sperm of *Aedes* must be stored in the thecae before they are capable of fertilizing the eggs.

Aedes spermatozoa *in vitro* are not obviously chemotactically directed to the vestibule, spermathecae, or their ducts, and thus are quite different from the sperm of the bed bug (Abraham, 1934). Since *Aedes* do not obviously pump sperm into their thecae, they are very different from *Ephestia* and *Plodia* (Norris, 1932) and the honey bee (Ruttner, 1956). The *Aedes* female never transports dead sperm to her thecae and is thus quite unlike the *Rhodnius* female which is said to be capable of doing this (Davey, 1958). The low degree of fertility following artificial insemination of both *Aedes* and *Drosophila* (Gottschewski, 1937) is in striking contrast to the high degree of fertility that is achievable by artificial insemination of the queen honey bee (Laidlaw 1944) and the bed bug (Davis, 1965). Indeed, in the queen honey bee, it is easy to obtain 100% fertility (Laidlaw, personal communication) and the artificially inseminated bed bug lays even more eggs than if she has been naturally mated (Davis, personal communication)!

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SUMMARY

1. Spermathecal filling in *Aedes aegypti* is not affected by changing the orientation of the intact female's terminalium.

2. Crushing the head of the female before forced-copulation tends to interfere with coitus. Decapitation of the female before forced-mating may lead to prolonged coitus but this does not necessarily result in insemination or spermathecal filling.

3. Crushing the head of the female during the act of forced-copulation does not interfere with coitus, insemination, or spermathecal filling. Crushing of the female's head immediately after coitus does not interfere with spermathecal filling.

4. Spermathecal filling can occur when the freshly inseminated female's terminalium is cut off into a drop of saline.

5. On a few occasions, spermathecae have been seen to fill with sperm in oil-covered saline whole mounts of the isolated reproductive system of a freshly inseminated female.

6. Sperm can ascend to the thecae during the time of oviposition, presumably between the intervals of egg depositions.

7. Spermathecal filling can occur in females which are externally immobilized by exposure to nitrogen, ether, carbon dioxide and cyclopropane.

8. Submerging freshly inseminated females in an ice bath at 4° to 7° C. for 10 to 30 minutes did not prevent spermathecal filling; at these temperatures the sperm are generally quite active. At 1° to 2° C., however, spermathecal filling is inhibited; at such temperatures sperm activity is greatly reduced or abolished.

9. Male *Aedes* can copulate with dead females and may deposit active sperm in the bursa but spermathecal filling does not occur.

10. Dyes injected only into the bursae of virgin females may be transported to one, two, or all three thecae, and to the common oviduct as far as the ampullae. But, dyes injected into the bursae of inseminated females are not transported to the thecae.

11. When seminal vesicle sperm with or without additives was injected into the bursa, only a few sperm reached the large theca in 13 (18.3%) of 71 cases.

12. When fresh ejaculates from donor bursae were injected into recipient virgin bursae, considerably better results were obtained in some cases and the sperm reached one or two thecae in 5 (35.7%) out of 14 cases.

13. The female is incapable of transporting dead sperm to her spermathecae. Highly active and oriented sperm artificially injected into the bursa generally do not fill the spermathecae.

14. The behavior of the sperm alone is not capable of explaining normal spermathecal filling. The role of the female during spermathecal filling is not clear.

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GONAD DEVELOPMENT AND DISCHARGE OF SPAWN IN OYSTERS OF LONG ISLAND SOUND

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Although the American oyster, *Crassostrea virginica*, is one of the most common invertebrates of our Atlantic and Gulf coasts, some aspects of its propagation are still not too well known. This is true, to a large extent, concerning the accumulation and release of its sexual products during the prespawning and spawning periods and, especially, the fluctuations in the quantity of gametes (or spawn) developed in different years.

The differences in the quantities of spawn accumulated by oysters in different years were considered important by several students of their propagation. For example, Prytherch (1929) and Needler (1931) maintained that intensity of setting of oysters was governed by the quantity of spawn formed prior to the beginning of spawning. In Europe, Cole (1951) and Korringa (1952) agreed that the numbers of larvae released—and these are roughly proportional to the quantity of eggs developed—control the intensity of the spatfall of the European oyster, *Ostrea edulis*. Although these views are not shared by all workers, it may be assumed, nevertheless, that the fluctuations in the annual quantities of spawn formed by oysters should be regarded as important not only in relation to the recruitment of oysters but also the recruitment of other groups, because eggs and larvae of oysters are eaten by many aquatic forms. For example, the young of a large group of pelagic fishes, especially the herring family (Clupeidae), feed on molluscan larvae (Thorson, 1946). Moreover, many other holoplanktonic animals, such as small crustaceans and their larvae, polychaete worms, . . . which also constitute an important part of the diet of fishes, in turn feed upon larval lamellibranchs.

Beds of *C. virginica* are found in every state along the Atlantic and Gulf coasts of the United States. These oysters reproduce by discharging their gametes into the surrounding water, where fertilization occurs. In many southern states oysters are extremely numerous and their spawning continues through most of the year (Hopkins, 1954). The fecundity of oysters is remarkable; a single Long Island Sound female, only about 4 inches long, may discharge 70 million eggs in one spawning (Davis and Chanley, 1956). In the years when oysters develop thicker gonads they discharge more eggs.

Because many oysters usually participate in spawning on natural beds, most of the discharged eggs become fertilized and develop into swimming larvae. The larval period may be long, sometimes extending several weeks, depending on the temperature and quantity and quality of food. Therefore, the larval population constitutes an important and, in some years, long-available food item for many other forms. Because of these and other considerations it is evident that the eggs and larvae of oysters are important parts of the food supply of other aquatic animals, and that the significance of fluctuations in the quantities of spawn from year to year cannot be questioned.

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Prytherch (1929) maintained that the prediction of time and intensity of setting of oysters in Long Island Sound could be made one month in advance, after careful analysis of several factors, one of which was the quantity of spawn accumulated by oysters. It was decided to undertake more detailed studies of this problem when we began to work in 1937—partly because Prytherch's conclusions were based on too short a period of observation, and principally because the literature contained little information on the accumulation of spawn in oysters, and its seasonal and year-to-year fluctuations. Our earlier observations in this field have already been reported (Loosanoff and Engle, 1940; Loosanoff and Nomejko, 1951a). Since then, however, much additional information has become available; this new material is the basis of the present article.

METHODS

The usual method of estimating the relative quantity of spawn in oysters is to measure the thickness of the layer of their gonadal tissue, which practically surrounds the body of a ripe individual (Loosanoff and Engle, 1942). This method is reliable, however, only if the following two rules are followed: first, oysters of about the same size must be used; and, second, the cut through the gonadal layer always must be made through the same part of the oyster. This point is especially important because the thickness of the layer varies along the anterior-posterior axis of the body (Fig. 1).

We adopted a uniform method, which consisted of sectioning the body of the oyster on the right side, along the line extending through the stomach on a level with the lower edge of the palps; we then measured the thickness of the gonadal layer in millimeters, with calipers. At the beginning of our work the samples at each station included 6 to 10 oysters, but commencing with 1944 each sample was composed of 10 individuals. The oysters were from $3\frac{1}{2}$ to $4\frac{1}{2}$ inches long.

Data on the thickness of the gonadal layer of oysters are available for the entire 20-year period of the studies (1937–56). The analysis is limited, however, to 1944–56 (although reference is made to material collected over the entire period) because observations in those years were made at the same 10 stations and by the same individual, thus eliminating certain personal errors that might otherwise have been introduced.

The 10 stations were along the north shore of Long Island Sound, between New Haven Harbor and Bridgeport Harbor, at 10-, 20- and 30-foot depths. Since the description and the chart showing the location of these stations were given in a recent volume of this journal (Loosanoff, 1964), they are not repeated here.

Observations on conditions of oyster gonads were usually begun in May, at the beginning of the spring gametogenesis, and continued until the majority of oysters had spawned completely. The thickness of the gonadal layer was measured at approximately weekly intervals early in the season, but more often—sometimes even daily—as the start of spawning approached. These frequent measurements made it possible to determine accurately the dates on which the maximal thickness of the gonadal layer was attained.

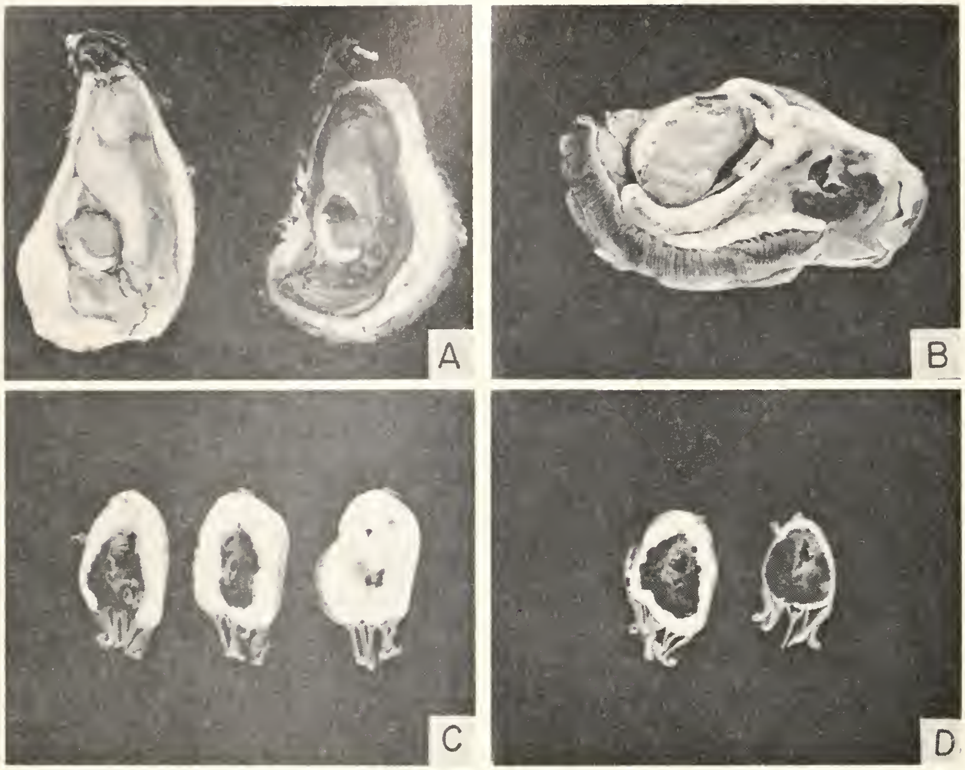

FIGURE 1. General condition of gonads of ripe and of spawned oysters. A. Ripe, unspawned adult oyster about 3½ inches long, with large, cream-colored gonad (left), and an individual with gonad almost completely discharged (right). B. Longitudinal section through body of adult oyster, showing well-developed, cream-colored gonadal layer surrounding digestive diverticula and stomach. C. Cross-sections through ripe oyster showing variation in thickness of the gonadal layer in different parts of the body. D. Cross-sections of two oysters at level of stomach: ripe individual with well-defined gonadal layer (left), and oyster that has discharged most of its spawn (right).

It should be mentioned that the maximal gonadal thickness referred to in this paper is not the average of the maximal values for each of the stations but the average of all the stations on the date of the highest grand average. Statistical analysis indicated, nevertheless, that these two measurements were similar, as is shown by the correlation between them of 0.97.

OBSERVATIONS

Gonads of *C. virginica* in northern waters undergo considerable changes during each year (Loosanoff, 1942). The fully developed gonad of an adult oyster is one of its most conspicuous organs and clearly distinguishes ripe individuals from unripe ones or from those that have already discharged all or most of their spawn (Fig. 1 A). The gonad is a virtually homogeneous, continuous mass surrounding the digestive tract and the digestive diverticula. Usually, a ripe gonad is cream-colored

(Fig. 1 B). The thickness of the gonadal layer, however, varies from one portion of the oyster body to another (Fig. 1 C). In spent individuals the layer is scarcely discernible (Fig. 1 D).

Histological studies of the gonadal tissue of Long Island Sound oysters have shown that during the cold season, when oysters are hibernating, their gonads are inactive (Fig. 2 A). The follicles are few and small and are found in the area between the body wall and the digestive gland, embedded in connective tissue. Nevertheless, the sexes can be distinguished even at this stage. In April, as the water temperature increases, spring gametogenesis is initiated and the follicles begin to enlarge, parallel with the increase in numbers of sex cells (Fig. 2 B). As the water becomes still warmer, proliferation and anastomosis of the gonadal follicles become more rapid, and late in May the follicles occupy a considerable space within the connective tissue. Toward the end of June most oysters in Long Island Sound are approaching ripeness (Fig. 2 C). At this time the larger part of the entire

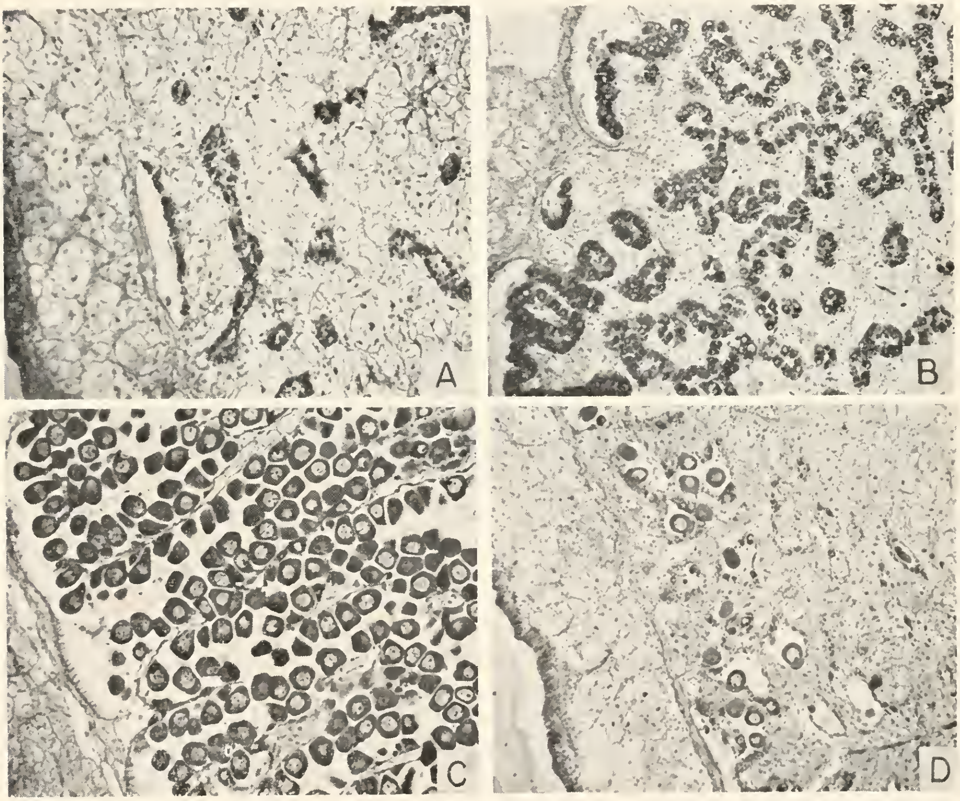

FIGURE 2. Seasonal changes in gonads of female oysters. A. Section of gonad in late spring, showing beginning of gametogenesis. Follicles, still in early stages of development, are surrounded by connective tissue (× 115). B. More advanced stage of ovogenesis, showing proliferation of follicles (× 115). C. Gonad containing eggs ready to be discharged (× 115). D. Gonad in advanced stage of spawning (× 75).

body is gonadal material, while the connective tissue, which so clearly predominated earlier in the season, has almost disappeared.

As is shown later, not all oysters begin to spawn at the same time, nor do they discharge their spawn at the same rate. Spawning of the same individual normally extends for many days or even weeks, although some oysters may discharge the gonads completely within a week after they begin to spawn.

Gonads in the late stages of spawning present a distinctive appearance, characterized by shrinkage of the follicles and invasion of a large number of phagocytic cells (Fig. 2 D). Reappearance of connective tissue also is normal during the late stages of spawning. A few unspawned eggs may be found in the shrinking follicles, but they will either be expelled later or be resorbed by phagocytes.

The date on which the maximal gonadal thickness was reached was determined for each station in each year from 1944 to 1956. At first it was thought that, instead of using the date of the single highest value, the date corresponding to the midpoint of the three highest values might be more reliable; when plotted, however, these two measurements were so close that it was decided to use the simpler measurement—the single date of the maximal gonadal thickness.

TABLE I

Dates maximal gonadal thickness was reached, and dates of various stages of discharge of the accumulated spawn by oysters of Long Island Sound, 1944–56

Year	Maximum thickness	Reduced to half maximum thickness	Days needed to reduce to half-thickness	50% of oysters more than half spawned	90% of oysters completely spawned
1944	7–21	8–4	14	8–5	8–24
1945	7–4	7–31	27	8–8	8–23
1946	6–27	8–1	35	8–1	8–28
1947	7–1	7–27	26	8–5	8–26
1948	6–30	8–3	34	8–5	9–4
1949	6–27	7–14	17	7–21	8–17
1950	7–19	8–20	32	8–16	9–6
1951	6–29	7–18	19	7–25	8–15
1952	6–30	7–16	16	7–31	8–20
1953	7–1	7–22	21	8–5	8–26
1954	6–30	7–24	24	7–28	8–24
1955	6–29	7–30	31	7–30	8–17
1956	7–6	8–16	41	8–22	9–5

Dates of maximal gonadal thickness for the individual stations and years varied from June 20 to August 11; the average date was July 7. The average of all the stations for each year had the earliest date of June 27 and the latest of July 21 (Table I); the grand average (all years) was about July 4. Usually, the oysters of the New Haven stations reached their maxima first, followed by the Milford stations a day or so later, and then by the Bridgeport stations. As could be expected, oysters at 10-foot depths reached their maxima several days earlier than those at depths of 20 or 30 feet.

During the entire period of observation, 1937–56, the earliest date at which the start of spawning was recorded was June 26, and the latest was July 3. Within the 13-year period, 1944–56, these dates were June 26 and July 1.

It was established that in some years spawning began before the average maximal gonadal thickness was attained. The explanation is that, although some oysters were already emitting small quantities of spawn, the majority of them were not spawning at all but were continuing, instead, to accumulate gonadal material for several more days.

During the 20 years the average maximal gonadal thickness varied from approximately 4.1 mm. in 1937 to 1.6 mm. in 1946 (Fig. 3). We suspect that the unusual gonadal thickness reported in 1937 may not be properly descriptive. Possibly, errors in recording occurred at the beginning of our studies, when the method was still being developed. Moreover, this high average was due, in part, to the unusually thick gonads recorded at one station, where they averaged almost 10.0 mm. The average maximum gonadal thickness for the 20 years was 2.4 mm.

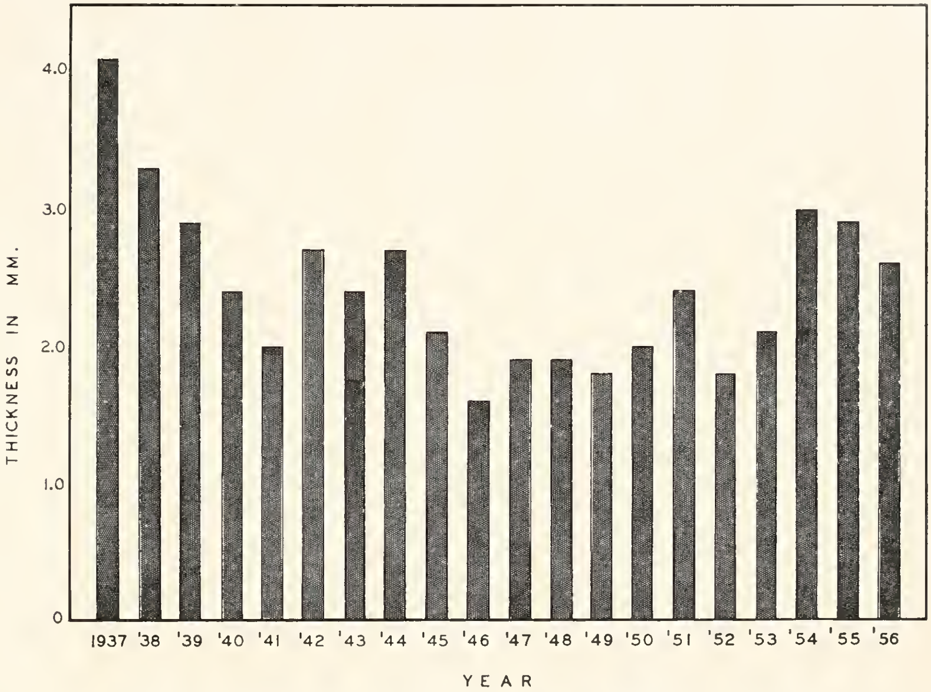

FIGURE 3. Average maximum thickness of gonadal layer of oysters, Long Island Sound, 1937–1956.

From 1944 to 1956 the maximal gonadal thickness varied from 1.4 mm. to 3.9 mm. for individual stations in different years, and the grand average was 2.2 mm. (Table II). An analysis of variance shows significant differences for years and depths but not for districts. Average yearly maxima ranged from 1.6 mm. (1946) to 3.0 mm. (1954).

A comparison of the three areas revealed that oysters from New Haven, especially from its three shallowest stations, attained somewhat greater gonadal thickness before the beginning of general spawning than oysters of the Milford or

Bridgeport areas (Table II). This difference was slight, only about 0.1 mm., and, moreover, station 8, located at the 10-foot depth in the Bridgeport area, contained oysters with gonads just as thick as at the best station in the New Haven area.

Oysters growing at the depth of 10 feet developed, in general, much thicker gonadal layers than did oysters in deeper water (Table II). The difference between the gonadal thickness of oysters from 10- and 30-foot depths was about 0.4 mm., which under the circumstances may be significant. This observation agrees with our earlier finding that oysters living in shallow water developed much larger quantities of spawn than those living at greater depths (Loosanoff and Engle, 1942).

Table II

Maximal thickness (millimeters) of gonad layer of oysters collected at the same 10 stations, at 3 different areas and depths, Long Island Sound, 1944–1956

Areas	Milford			New Haven				Bridgeport			Average
Stations	1	2	3	4	5	6	7	8	9	10	
Depth (feet)	10	20	30	10	10	20	30	10	20	30	
Year											
1944	2.0	2.5	2.0	2.0	2.0	3.0	3.0	4.5	3.0	2.8	2.7
1945	2.5	2.0	1.8	1.9	2.6	2.4	2.2	2.2	1.8	1.4	2.1
1946	2.1	1.7	1.1	1.9	2.0	1.7	1.7	1.5	1.3	1.1	1.6
1947	1.7	2.2	2.0	1.8	2.1	2.2	1.8	1.6	1.7	1.7	1.9
1948	2.3	2.1	1.3	1.9	2.6	2.5	1.8	2.1	1.5	1.0	1.9
1949	2.1	2.2	2.0	1.6	1.5	1.6	1.7	1.5	2.2	1.9	1.8
1950	1.8	1.8	2.1	2.6	2.6	2.5	1.7	1.8	1.4	2.0	2.0
1951	2.5	2.1	2.1	2.2	2.5	2.3	2.4	2.9	2.6	2.5	2.4
1952	1.8	1.8	1.0	2.4	1.8	2.2	1.1	2.5	1.6	1.3	1.8
1953	2.6	2.3	1.3	2.3	2.6	2.5	1.7	2.4	1.8	1.5	2.1
1954	3.2	3.1	2.6	3.0	2.8	3.4	3.0	3.4	3.1	2.9	3.0
1955	2.9	2.9	2.7	3.0	3.1	3.2	2.8	2.7	3.1	2.9	2.9
1956	2.5	2.8	2.9	2.8	2.3	2.6	2.7	2.7	2.5	2.4	2.6
Station average	2.3	2.3	1.9	2.3	2.4	2.5	2.1	2.5	2.1	2.0	2.2
Area average	2.2			2.3				2.2			
Depth average	10 ft 2.4			20 ft 2.3				30 ft 2.0			

We still do not know why shallow-water oysters develop more spawn. The temperatures at the three depths varied only one or two degrees, a difference not significant enough to affect the metabolic processes of the oysters, especially their rate of feeding. It has been demonstrated that, in the wide temperature range from about 16° to 28° C., the rate of water pumping in oysters showed no great fluctuation (Loosanoff, 1958). The oysters may have accumulated more spawn in shallow water because the phytoplankton organisms they use as food were more abundant at that depth.

The increase in thickness of the gonadal layer prior to the state of ripeness and the release of the material during active spawning are relatively slow processes.

This fact is well illustrated in Table III, which shows average thicknesses of gonadal layers recorded at each sampling station during the entire 1956 season. These observations were begun before the gonads were thick enough to measure and were continued until they were almost spent.

The first measurable gonads were found about June 7, when the thickness of the layer varied from 0.1 to 0.4 mm. By the beginning of July, however, the layer at some stations was near or at the maximum. Thus, the major accumulation of spawn required 3 to 4 weeks.

After the beginning of spawning the thickness of the gonadal layer remained at about the same level for 12 or 14 days, but then it began to decrease (Table III).

TABLE III

Average thickness (millimeters) of gonad layer of oysters at each of 10 sampling stations, and average of all stations. Long Island Sound, May 31–September 5, 1956. Sample at each station was 10 oysters

Areas	Milford			New Haven				Bridgeport			Average
Stations	1	2	3	4	5	6	7	8	9	10	
Depth (feet)	10	20	30	10	10	20	30	10	20	30	
Date											
May 31	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
June 7	0.0	0.0	0.10	0.35	0.40	0.35	0.15	0.0	0.10	0.10	0.16
13	0.50	0.75	0.60	1.55	1.45	1.60	0.65	0.0	0.40	0.25	0.84
19	0.80	1.40	1.40	1.70	1.70	1.55	1.35	1.15	0.90	0.70	1.27
28	1.95	1.95	1.80	2.70	2.80	2.15	2.10	2.15	1.95	1.80	2.14
July 2	2.25	1.90	2.25	3.45	2.30	2.15	3.15	2.75	2.05	2.35	2.43
6	2.50	2.80	2.85	2.75	2.30	2.55	2.70	2.70	2.50	2.40	2.60
10	2.75	2.60	2.65	2.60	2.35	2.45	2.65	2.45	2.55	2.65	2.57
18	2.70	2.35	2.20	2.35	2.15	3.05	2.75	3.00	2.70	2.70	2.60
25	2.65	1.95	1.55	2.10	1.40	2.00	2.55	3.15	2.35	1.85	2.16
Aug. 1	2.00	1.70	2.00	1.35	1.25	1.40	2.15	2.35	2.00	1.90	1.81
8	1.25	2.40	1.80	1.65	1.45	1.50	1.70	3.35	2.65	1.80	1.96
15	0.85	2.65	1.80	0.95	0.55	0.75	2.00	2.35	1.25	1.65	1.48
22	0.30	1.45	0.90	0.75	0.20	0.25	0.50	0.65	1.00	0.90	0.69
29	0.0	0.90	0.50	0.10	0.0	0.40	0.20	0.25	1.45	1.25	0.51
Sept. 5	0.0	0.05	0.15	0.05	0.0	0.0	0.0	0.0	0.20	0.0	0.05

Early in August the decrease was evident at all stations and depths. By late August and early September the gonads at some stations were so spent that they could not be measured.

The rate of spawn discharge varies considerably in different years. One criterion employed was the determination of the date when the maximum gonadal thickness was reduced to about half its original size. The earliest date this occurred was July 14, 1949, and the latest, August 20, 1950 (Table I). Thus, these two dates were more than a month apart, while the mean date for the 13-

year period was July 27. The number of days needed in different years to achieve this reduction varied from 14 in 1944 to 41 in 1956 (Table I).

The dates on which more than 50% of the oysters had passed the half-spawned stage ranged from July 21 in 1949 to August 22 in 1956 (Table I). The mean date was August 3, which may therefore be considered as approximately the mid-point of the spawning season of Long Island Sound oysters.

Another criterion used in studying the progress of the spawn discharge was establishment of the date at which 90% of the total oyster population was completely spawned. This date varied from August 15 in 1951 to September 6 in 1950, and the mean was August 25.

Further details on the rate of accumulation of sexual products, attainment of maximal thickness, and release of spawn by Long Island Sound oysters living at three different depths are given in Figure 4, which shows these conditions for 1946, 1950 and 1956. The year 1946 was characterized by relatively poor gonad development—maximal average thickness, only 1.6 mm. The gonadal layer of shallow-water oysters, however, was approximately 1.9 mm. thick. This figure was reached about June 27, but after that date the gonadal thickness of these oysters began to decrease rapidly and soon fell below the measurements recorded at the other two depths, especially at 20 feet.

In 1950 the gonadal layer developed rather rapidly, reaching almost complete fullness on June 28 (Fig. 4). Unlike 1946, however, the gonadal thickness at all three depths was about the same for a considerable period of time. As in 1946, however, the shallow-water oysters discharged their spawn sooner than those at the other two depths. The period when the average gonadal layer was at or near its maximum extended even longer than in 1946. Regardless of the greater accumulation of spawn in 1950, the spawning was nearly completed by September 6, only a week later than 1946.

The year 1956 was representative for the seasons when accumulation and discharge of gonad material by the oysters of all three depths progressed at about the same rate, and the quantities of spawn accumulated were nearly the same. The only appreciable difference was during the latter part of the spawning season, when the gonads of shallow-water oysters became spent somewhat earlier than did those of oysters at 20 or 30 feet. Moreover, development of gonads was better in 1956 than in 1946 and 1950; the average maximal thickness was 2.6 mm., approximately 0.4 mm. greater than the average for the entire period, 1944–56.

Loosanoff and Engle (1942) showed that individual oysters of Long Island Sound do not begin to spawn at the same time nor do they discharge their spawn at the same rate; ripe but unspawned individuals can be found as late as 6 weeks after the beginning of spawning, whereas some oysters may complete their spawning within the first week of the season. On the basis of these observations they concluded that, "It is possible that these individuals represent a race physiologically different from other local oysters whose spawning periods extend about two months." It was suggested that these individuals are descendants of the race of oysters brought to Long Island Sound from northern waters, where the spawning season is considerably shorter than in Long Island Sound.

Some other oysters, on the other hand, whose gonads late in summer were still thick, well-developed, and possessed ripe gametes, possibly represented races that originated in the process of mixing oysters from many different points along the Atlantic coast, or were from southern races that required longer periods and higher temperatures than northern oysters for complete maturation and discharge of their sex cells. This conclusion was soon verified by experiments which demonstrated that oysters of different geographical regions display different temperature require-

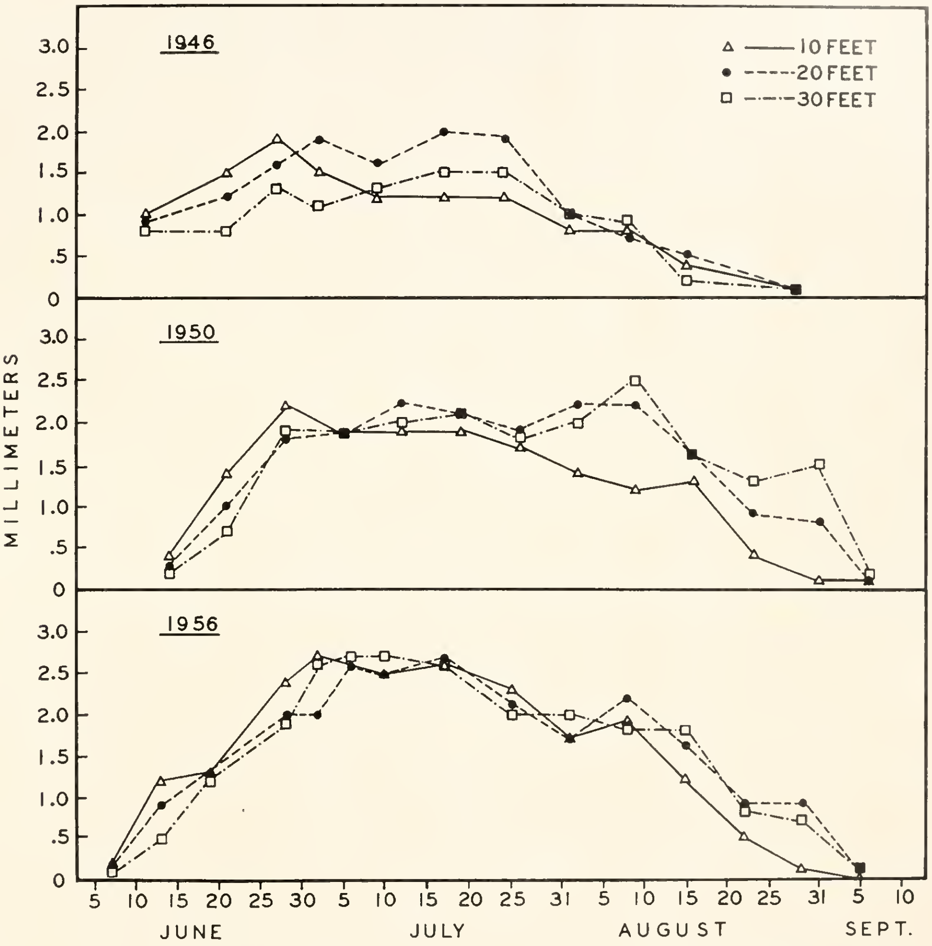

FIGURE 4. Average thickness of gonadal layer of oysters living at three different depths in Long Island Sound in 1946, 1950, and 1956.

ments for gonad development and spawning (Loosanoff and Nomejko, 1951b). A few months earlier Stauber (1950) offered an excellent theoretical discussion, which he based primarily on the observations of Hopkins (1931) and Loosanoff (1932), on the problem of physiological races within the general population of *C. virginica.*

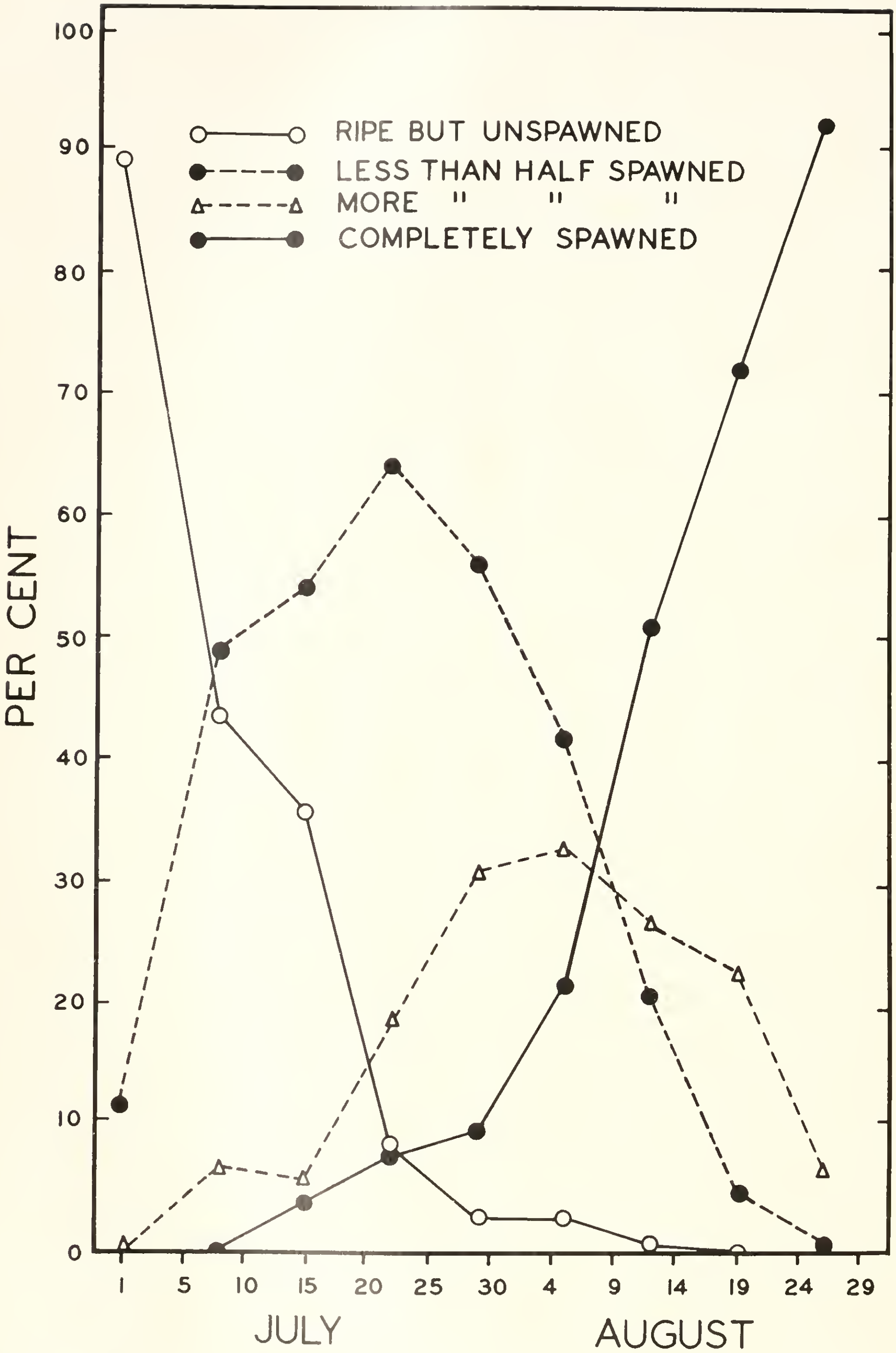

FIGURE 5. Percentage of ripe, partially spawned, and completely spawned individuals composing the oyster population at depths of 10 to 30 feet, Long Island Sound, July–August 1953.

The present studies fully support our earlier observations. Let us take 1953 as an example; when spawning began on July 1, only about 11% of the oysters showed partly discharged gonads, and the remaining 89% were still ripe but unspawned (Fig. 5). A week later only 44% of the oysters remained ripe but unspawned, 49% were less than half spawned, and the remaining 7% were more than half spawned. No completely spawned oysters were found on this date. They appeared first in samples collected on July 15, when about 4% of the oysters examined were completely spent. On that date, therefore, four different groups of oysters, namely, ripe but unspawned, less than half spawned, more than half spawned, and completely spawned, were found among the general population. From that date onward the percentage of completely spawned oysters increased rapidly, while the percentage of unspawned individuals became smaller and smaller until, on August 19, no unspawned individuals remained.

The observations in Long Island Sound have demonstrated, therefore, that even when the so-called "mass spawning" of oysters occurs, it does not involve the entire population, or sometimes even the majority. These field observations are strongly supported by the laboratory experiments of Davis and Chanley (1956), which show that not all oysters may be induced to spawn at the same time, and that, in general, spawning of an individual oyster is not a short-term process, during which the entire supply of sex cells is discharged, but, rather, a lengthy physiological effort which may continue for several weeks before an oyster is entirely spent.

As already mentioned, it is possible that in some aquatic basins the numbers of eggs and larvae of oysters may affect the survival and growth of planktonic organisms that feed on them. In that event, the ability to predict the total production of spawn of oysters and other lamellibranchs may be an important step toward the understanding of fluctuations in aquatic populations, including those of certain fishes. To ascertain such a possibility the data on gonad development of the oysters from 1944 to 1956 and the data on various biological and environmental variables recorded during the same period were subjected to conventional statistical analysis and to the computational techniques developed by the University of California at Los Angeles for biomedical programs for statistical analysis of biological data. All computations were made on a Burroughs B-5500 computer.

In evaluating the factors of the environment, which could be important in determining the quantities of spawn developed by the oysters in different years, two variables showed high correlations with maximal gonadal thickness. One was the departure of the average daily air temperature from normal during the period from September 1 to November 15 of the preceding year. This variable, to be indicated here as X_{at}, gave a correlation coefficient of 0.64. The second variable, X_s, the departure of percentage of possible sunshine from normal for the period indicated above, and maximal gonadal thickness gave a correlation of −0.80.

Since the intercorrelation of X_{at} and X_s was found to be −0.45, some improvement in prediction seemed possible by using a multiple correlation rather than the best single variable, air temperature. Accordingly, a multiple regression equation was computed to find the weighting of the two variables which would give the best prediction of the maximal gonadal thickness, X_{gt}':

$$X_{gt}' = -0.2021\, X_{at} + 0.0110\, X_s + 2.778.$$

The multiple correlation between the observed maximal gonadal thickness, X_{gt}, and the maximal gonadal thickness, X_{gt}', predicted from the weighted combination X_{at} and X_s, was 0.85.

Since N, the number of years during which observations were made, was 13 and the number of predictor variables was 2, the value of the multiple correlation must be at least 0.78 for statistical significance at the 0.01 probability level. The actual multiple correlation of 0.85 exceeds this value; a good functional relationship between the two variables, *i.e.*, the predicted thickness and the observed thickness of the gonadal layer, is shown (Fig. 6). This relationship indicates that cool air and bright days during the preceding fall favor a greater accumulation of spawn by oysters during the succeeding summer.

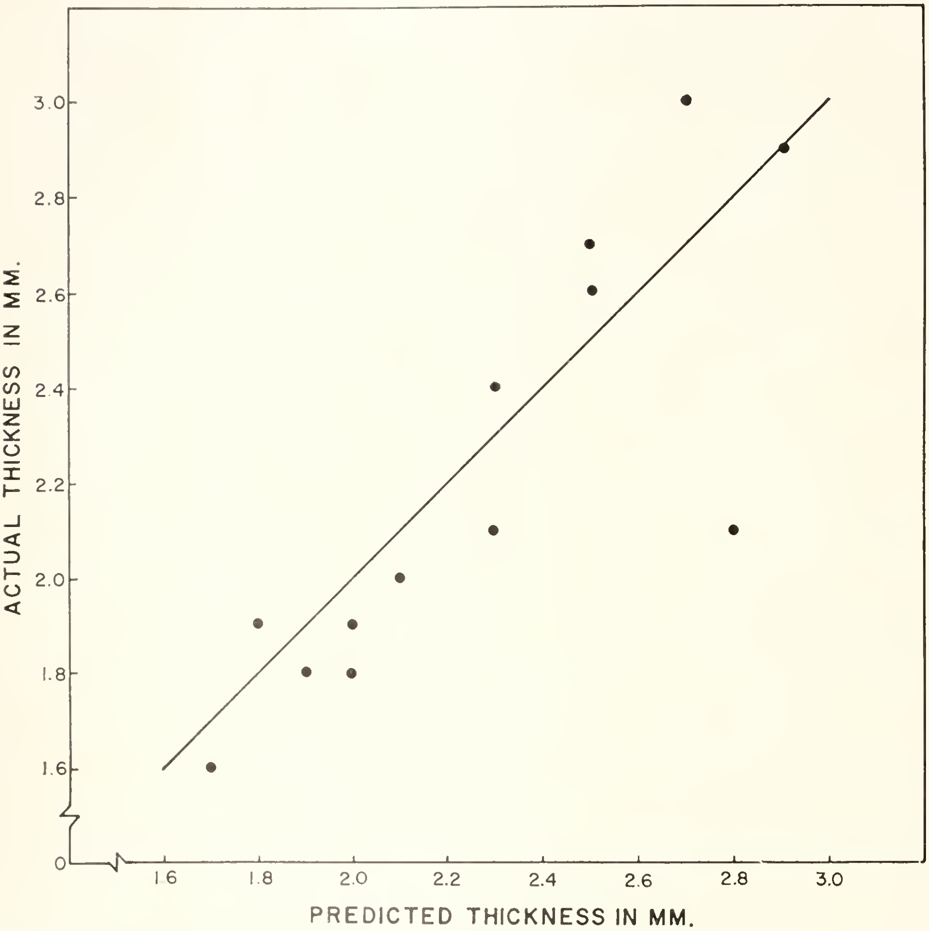

FIGURE 6. Relationship between actual and predicted maximal gonadal thickness in oysters of Long Island Sound over the 13 years, 1944–1956.

Use of the multiple regression equation is always subject to criticism on the grounds that it capitalizes on high individual correlations which may be due to chance alone. If a very large number of potential predictor variables are tried, as in our study, some should yield statistically significant correlations by chance alone. It is possible, therefore (though not highly probable), that the apparently close relations between maximal gonadal thickness and the two environmental variables used for the prediction resulted merely by chance and do not represent true relationships.

The answer to this kind of criticism is not a statistical one but a biological explanation as to the meaning of this apparent relation. I offer the explanation that the combination of relatively low air temperature and bright, clear days between September 1 and November 15 of the preceding year is a favorable combination of environmental factors for the growth and reproduction of microplanktonic organisms eaten by oysters. At this time of the year the oysters of Long Island Sound store in their bodies most of the reserve material which, during gametogenesis the next spring, is converted into gonads. Thus, the more glycogen and other material occumulated the preceding fall, the greater is the thickness of the gonadal layer the following summer.

Since many examples are known in aquatic biology whereby apparently highly significant relationships between two or more variables failed to behave in the same manner soon after they were reported, I realize that the validity of my conclusions should be tested by further observations. It should be interesting, also, to ascertain whether my suggestions concerning the possibility of long-range prediction of the quantity of spawn developed by oysters of Long Island Sound are applicable to *C. virginica* of other geographical areas, and also to other species of oysters, especially of the genus *Crassostrea,* which was introduced to our Pacific coast and is the main commercial species of oysters in the waters of Japan.

I wish to express my thanks to Mrs. Barbara J. Myers for the statistical analysis of the data, to Charles Nomejko for his assistance during these studies, and to Mrs. S. Webb for her help in preparation of the manuscript.

SUMMARY

1. During the 13 years, 1944–56, the average maximal gonadal thickness of oysters from Long Island Sound was attained between June 27 and July 21; the grand-average date was about July 4.

2. Gonads of oysters living at 10-foot depths reached their maximal thickness several days earlier than oysters living at stations 20 or 30 feet deep. Oysters of the New Haven area reached their maxima a day or so earlier than individuals of the Milford and Bridgeport stations.

3. Between 1937 and 1956, the earliest date at which spawning started was June 26, and the latest, July 3. In some years spawning began before the maximal gonadal thickness was attained.

4. Over this same 20-year period the average maximal gonadal thickness varied from approximately 1.6 mm. to 4.1 mm. and averaged 2.4 mm. In 1944–56, when observations were made at the same 10 stations, maximal thickness varied from

1.4 mm. to 3.9 mm. for individual stations, and had a grand average of 2.2 mm. Average annual maxima during this period ranged from 1.6 mm. to 3.0 mm.

5. Oysters growing at depths of 10 feet developed thicker gonads than did individuals in deeper waters.

6. The rate of spawn discharge varied considerably in different years. The earliest date at which the maximal gonadal thickness was reduced to about half its original size was July 14, and the latest, August 20. The number of days needed in different years to achieve this reduction varied from 14 in 1944 to 41 in 1956.

7. The days on which more than 50% of the oyster population was in more-than-half spawned stage ranged from July 21 to August 22. The mean date for this event, August 3, represents the approximate mid-point of the spawning season in Long Island Sound.

8. The date at which 90% of the oyster population was completely spawned varied in different years from August 15 to September 6, and the mean was August 25.

9. Recent observations support the previous conclusion that the oyster population of Long Island Sound is not homogeneous but a mixture of many physiological races.

10. Statistical analysis of the data suggests that the combination of relatively low air temperatures and bright, clear days between September 1 and November 15 of the preceding year is a favorable combination of environmental factors responsible for a larger accumulation of gonadal material the following summer. An explanation of this phenomenon, based on biological considerations, is offered.

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FURTHER STUDIES ON THE EFFECT OF PHENYLTHIOUREA ON PIGMENTATION BY MELANIN IN AMPHIBIANS

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The present work is essentially an extension of our earlier studies (Millott and Lynn, 1954) on *Eleutherodactylus* to the young stages of other amphibians.

The familiar brown or black coloration of amphibian tadpoles is usually attributed to melanin, despite the fact that aside from our former investigations on *Eleutherodactylus*, little attempt has been made to characterize the pigment histochemically. It has been shown, however, that certain parts of the larva of *Xenopus* possess the power to oxidize "dopa" (Sims, 1962).

Our former investigations have shown that the skin and retina of *Eleutherodactylus* lose their pigmentation when embryos are treated with phenylthiourea and regain it on cessation of treatment. This effect is usually attributed to the action of phenylthiourea on the process of melanin formation, because phenylthiourea does not affect the pigment directly, so that loss of color implies that elimination of pigment must occur, which only becomes evident when the normal channel of replacement is impeded.

There are significant gaps in our knowledge. First, in amphibians where black pigment is widespread, the extent to which the pigmentation in many sites can be affected by phenylthiourea is incompletely known. Second, the routes by which pigment is eliminated have not been shown, although it seems reasonable to suppose that pigment could be lost from the epidermis by desquamation. Third, the evidence of turnover (Millott and Lynn, 1954) was obtained from an amphibian with direct development, and in any event little information is at hand concerning the length of time for which such a process persists. Finally, although it has been assumed that phenylthiourea acts only on melanogenesis, if turnover occurs, the possibility of an effect of this substance on excretion of the pigment needs to be considered.

Some relevant findings in *Xenopus* have been reported by Sims (1961), who found that it was not possible to affect pigmentation in normal development even by six weeks' treatment with phenylthiourea, if treatment did not begin until stages 46/47 (Nieuwkoop and Faber). The substance was therefore considered to be effective in normal development only during the early stages, although its effectiveness persisted in regenerating tails of embryos up to stages 46–49. During this time and in such areas, the melanophores were supposed still to be accumulating their pigment, so that the effect of the drug could be detected by its blocking action on melanogenesis. The gradual loss of effectiveness was held to indicate the absence of any turnover of the pigment. This interpretation was substantiated by experiments using "dopa," which was converted to melanin by melanophores in normal and regenerating tissue, only during their formative stages and not when they were already loaded with pigment.

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Lack of turnover is not easily reconciled with the appearance of pigment in certain situations in amphibian larvae such as the gut and kidney tubules. We therefore decided to examine the pigmentation of such areas more carefully and particularly to determine what effect phenylthiourea had on it.

Materials and Methods

The larvae of *Xenopus laevis, Rana pipiens, Rana temporaria* and *Ambystoma mexicanum* were used. For convenience, most of the experiments were performed on *Xenopus*, but because of their more widespread pigmentation, certain stages of *Ambystoma* and the two species of *Rana* were used for comparison.

Developing embryos were kept in shallow dishes at room temperature, except for *Ambystoma*, where they were reared at 10° C. They were fed on a suspension of dry nettle powder. For control purposes (see below) a few were fed on "Complan" milk powder or deprived of food.

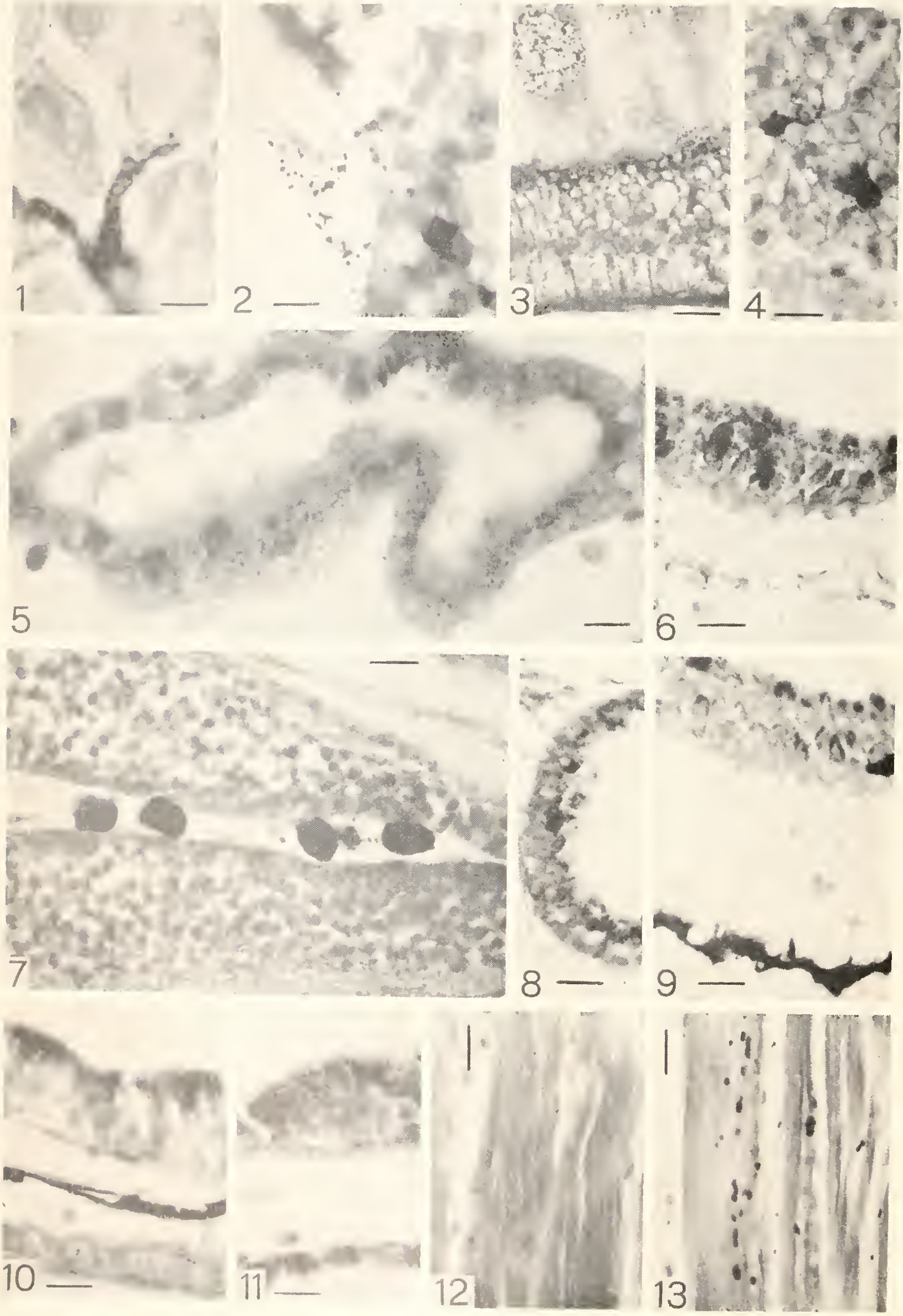
Sufficient phenylthiourea to make a final concentration of 0.01% was added to some of the dishes and in all cases the embryos in such solutions were compared with others kept in separate dishes and reared without phenylthiourea. The dishes were kept side by side to ensure uniform conditions, and their fluid contents were changed daily. In *Xenopus* an extensive series of experiments was performed in which treatment was begun at various stages ranging from 25 to 56, with experimental animals and controls being fixed at successive times over a period of about four weeks after the beginning of treatment. Another series with *Xenopus* consisted of raising animals in which treatment was begun at stage 26 and returning them to water at various times ranging from three to 31 days after the beginning of treatment. With the other amphibians, similar but briefer experiments were performed, mainly confined to the first three weeks after hatching.

Larvae were fixed in 10% formol and stained by Mallory's triple method, or, more rarely, in Ehrlich's hematoxylin.

Identification of the Pigment

As already emphasized (Millott, 1953), the identification of melanin, when the quantities of pigment available are so small, is extremely difficult. Happily it is now possible to subject the pigment to additional tests, thus extending those previously used by Millott and Lynn (1954). In addition to the latter tests, the pigment in sections of tadpoles of each species studied, with the exception of *Ambystoma*, was subjected to the Nile Blue method which differentiates melanins from lipofuscins (Lillie, 1956), and to the action of $M/10$ sodium hydroxide, 10 M formic acid, concentrated sulfuric acid and potassium borohydride, which serve to differentiate the pigment from ommochromes. When insufficient material was available for all of these tests, a shorter method of diagnosis had to be used, the pigment being subjected to 10 M formic acid for 4–60 hours, 50 vol. hydrogen peroxide for 48 hours and to Lillie's Nile Blue method.

In all of these tests the pigment behaves like melanin (see Lison, 1953; Fox and Vevers, 1960). It is extremely resistant to solvents and does not dissolve in sodium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Figures 1–13.

hydroxide or in formic acid after 24 hours, or in concentrated sulfuric acid. It is bleached by 24 hours' treatment with bromine and 48 hours' treatment with 50 vol. hydrogen peroxide. It is not reduced by potassium borohydride, but readily reduces ammoniacal silver nitrate. It usually appears dark green after the Nile

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FIGURE 1. *Rana pipiens.* Transfer of melanin from a melanophore to an epidermal cell. From a larva 19 days after hatching. Two dark processes of a melanophore insinuated among epidermal cells appear on the right. One process makes intimate contact with an epidermal cell into which it is apparently transferring two granules of pigment. Fixed, 10% formol; stained, Mallory's triple. Scale = 5 μ.

FIGURE 2. *Xenopus laevis.* Discharge of melanin into the ventricle of the diencephalon of a larva at stage 51. On the right, melanin is seen in the lateral wall of the diencephalon, from which secretion containing pigment may be seen passing off. The preparation has been subjected to Masson's argentaffin test for melanin. Fixed, 10% formol; stained, Mallory's triple. Scale = 8 μ.

FIGURE 3. *Xenopus laevis.* Discharge of melanin into the lumen of the intestine of a larva at stage 45. Granules of pigment discharged with secretion from the free border of the intestinal epithelium are visible on the right. The preparation was treated with 10 *M* formic acid for 24 hours. Fixed, 10% formol; stained, Mallory's triple. Scale = 12 μ.

FIGURE 4. *Rana pipiens.* Portion of liver, showing melanin in hepatic cells of a larva 19 days after hatching, which had been treated with 0.01% phenylthiourea since hatching. Fixed, 10% formol; stained, Mallory's triple. Scale = 15 μ.

FIGURE 5. *Rana pipiens.* Pronephric tubule of the same larva as that used for Figure 4, showing granules of melanin in walls. Fixed, 10% formol; stained, Mallory's triple. Scale = 12 μ.

FIGURE 6. *Rana temporaria.* Section through the skin from the snout of a larva 11 days after hatching, showing the effect of 9 days' treatment with 0.01% phenylthiourea begun two days after hatching. Note that the pigment in the dermal melanophores has almost disappeared (*cf.* Fig. 9), but that in the epidermal cells persists. Fixed 10% formol; stained, Mallory's triple. Scale = 12 μ.

FIGURE 7. *Xenopus laevis.* Frontal section of the anterior region of the neural tube in a larva at stage 41. The larva had been treated with 0.01% phenylthiourea for 7 days beginning at stage 26. Note the large aggregates of melanin in the central canal. Fixed, 10% formol; stained, Mallory's triple. Scale = 5 μ.

FIGURE 8. *Rana temporaria.* Portion of the membranous labyrinth from the same larva as used for Figure 6. Note the persistence of pigmentation after treatment with phenylthiourea. Fixed, 10% formol; stained, Mallory's triple. Scale = 20 μ.

FIGURE 9. *Rana temporaria.* Control for Figure 6, showing a similar section through the skin of a larva of the same age as that in Figure 6, but the larva had not been treated with phenylthiourea. Note the deeply pigmented dermal melanophores and the pigment within the epidermal cells. Fixed, 10% formol; stained, Mallory's triple. Scale = 12 μ.

FIGURE 10. *Rana temporaria.* Section of a portion of the intestinal wall and adjacent body wall of a larva 11 days after hatching. Note the deeply pigmented parietal peritoneum and the pigment in the intestinal epithelium (uppermost in the photograph). Fixed, 10% formol; stained, Mallory's triple. Scale = 12 μ.

FIGURE 11. *Rana temporaria.* Similar section to that shown in Figure 10, from a larva of the same age, but which had been treated for 9 days with 0.01% phenylthiourea, beginning two days after hatching. Note that the pigment in the parietal peritoneum has disappeared, but that in the intestinal epithelium persists. Fixed, 10% formol; stained, Mallory's triple. Scale = 12 μ.

FIGURE 12. *Rana pipiens.* Striated muscle and connective tissue of myotomes, as they appear in a larva 19 days after hatching. Fixed, 10% formol; stained, Mallory's triple. Scale = 20 μ.

FIGURE 13. *Rana pipiens.* Striated muscle and connective tissue of myotomes, as they appear in a larva 19 days after hatching, which had been treated from the time of hatching with 0.01% phenylthiourea. Note the deposition of melanin. Fixed, 10% formol; stained, Mallory's triple. Scale = 20 μ.

Blue method, though the test is sometimes difficult to apply where the pigment is dense.

The results of some of the tests are seen in Figures 2 and 3.

Thus, within the limitations imposed by the methods of identification which can be used, the black pigments in the various locations noted below and in all the species examined with the exception of *Ambystoma*, to which the tests were not applied, may be described as melanin.

The Distribution of Melanin

Melanin quickly becomes widely distributed in all the larvae examined. In general, it becomes progressively more widespread with age, and in *Xenopus* it shows a steadily widening distribution up to the onset of metamorphosis (the latest stage studied).

An indication of such distribution in *Xenopus* has already been given by Sims (1961) who describes it in melanophores in the dermis, over the head, the dorsal aspect of the trunk, around the nasal organs, and over the superficial surface of the axial muscles of trunk and tail. He also describes it among the fibers of these muscles, in the meninges, the parietal peritoneum, on the pronephros, on the lungs, and in the mesentery of the gut. It is described in the eye, the medial wall of the otic capsule, in the walls of some blood vessels, in the liver and in the intestinal wall.

In general, our studies have confirmed this distribution but, impressive though this list may be, it is incomplete, for at various stages we find pigment in the walls and central canal of the neural tube (Fig. 7), in the walls of the brain and in the cerebral ventricles (Fig. 2), in the walls of the membranous labyrinth (Fig. 8), in the pancreas, spleen, epidermis, and notably in the epithelial lining and lumen of the gut (Fig. 3). Furthermore, the pigment is even wider in distribution in the two species of *Rana* and in *Ambystoma*, where it is also found in the gills, in cartilage corpuscles, in both pro- and mesonephros, in the wall of the heart and major blood vessels, olfactory epithelium and associated with blood corpuscles. In these latter species, melanin is therefore ubiquitous, but certain features of its disposition deserve amplification.

Thus, the pigmentation of some of the foregoing structures may arise in two ways: by the presence of granules of melanin in the cells or in the cavities of the structure itself, and by the presence of pigment-laden processes of melanophores which penetrate the structure from outside. We are concerned here with pigmentation of both types, but we regard the latter type as adventitious and appropriate to the structure housing the nucleated central body of the melanophore. Unfortunately the distinction has not always been made. In *Xenopus*, aside from the pigment in situations such as the eye, Sims (1961) seems to be concerned mostly with that in melanophores, and indeed mentions their penetration in the case of the intestine, but he does not make clear to what extent pigmentation is due to this cause in other situations. It is very clear from our studies that even in *Xenopus*, there is much pigment that is not contained in melanophores.

A case in point exists in the gut, where melanin can be seen near the free border of cells composing the epithelial lining from which it appears to be cast off with secretion into the lumen (Fig. 3). It might be argued that this represents the absorption of dark granules from the food mass, but it is unlikely that such appearances are being misinterpreted, for not only do the dark granules respond histochemically like melanin, but they are contained in strands of coagulum associated with the free border, that are so characteristic of fixed preparations of actively secreting epithelia. Further, they are to be found thus in tadpoles that have been starved, or reared only on milk powder. It is equally unlikely that the epithelial melanin is segregated egg pigment, for there is far more of it than can be found in the organizing gut of early embryos.

A more striking instance is seen in the central nervous system, where melanin is discharged from the walls, particularly in the brain (Fig. 2), and accumulates in the ventricles and central canal of the neural tube (Fig. 7), forming aggregates that are sometimes so large as to fill the lumen. In *Xenopus,* at least, they do not persist but tend to disappear between stage 52 and metamorphosis.

The pigment in the myotomes is partly contained in the processes of melanophores which penetrate from the dermis, but much occurs in the muscle fibers as well as in the surrounding connective tissue and in myoblasts. The latter type of pigment is well represented in *Ambystoma* and *Rana temporaria.*

Much the same applies to the pigmentation in the pro- and mesonephroi. In *Xenopus,* it is entirely due to processes penetrating from melanophores in the investing peritoneum or dermis, but in the other species, granules of melanin also appear in the walls of the tubules (Fig. 5).

The situation in the epidermis is markedly different in *Xenopus* from that of the other species examined here. In *Xenopus* epidermal pigment is rare and found only in the oral sucker and in the tip of the tail between stages 25 and 36. In the other species it is abundant in certain areas such as the snout, and consists of finely granular masses disposed in the cytoplasm so as to form caps of pigment over the nucleus, on the outer sides of the epidermal cells (Figs. 6 and 9). In addition, the epidermis is penetrated by the processes of dermal melanophores. Scrutiny of such processes reveals here and there suggestive appearances such as that in Figure 1, where granules seem to be passed into the epidermal cells from melanophore processes insinuated among them, thus confirming the earlier observations made by Ehrmann (1885).

It thus appears that melanin is being eliminated, yet larvae do not become paler with age, which means that it is constantly being formed, so that turnover occurs despite assertions to the contrary (Sims, 1961, 1962), though it is not known for how long it persists. At the same time the pigmentation of some areas is transient in character so that there is a temporal as well as a spatial pattern of pigmentation. Both patterns show some significant features, especially when the effect of phenylthiourea on pigmentation is studied.

THE DEVELOPMENT OF PIGMENTATION AND THE EFFECT OF PHENYLTHIOUREA

The account of development which follows is the result of observations on *Xenopus.* The effect of phenylthiourea was also studied in this animal, but for purposes of comparison, especially in regions where pigment is scarce, the effect of the substance was determined at certain stages in *Ambystoma* and the two species of *Rana.*

Since phenylthiourea not only affects pigmentation but also retards development, controls of two kinds are necessary to assess its effect. Those of the first type were larvae derived from the same parents and of the same age, but reared in water without phenylthiourea. Those of the second type were larvae reared without phenylthiourea, but otherwise under the same conditions, and sectioned at a stage of development equivalent to that of the treated individuals at the end of their sojourn in phenylthiourea.

The earliest larvae examined were those between stages 24 and 29 in which the definitive gut was organizing. Pigment was present, notably in the sucker and organizing epidermis, but small amounts were present in scattered clumps among yolk spherules in the lumen of the foregut and in the neural tube. The pigment differs from that which appears later in that most of it (aside from that in the sucker) lies at the surface of cells and in interstitial spaces. It is unaffected by two days' treatment with phenylthiourea. There is thus no evidence that it is still being formed, and it appears to be egg pigment redistributed by morphogenetic movements and seemingly eliminated so as to drain into spaces such as the foregut and neural tube. It is far too small in amount to account for the pigment that accumulates later in these cavities.

Melanin appears rapidly between stages 25 and 36 in the eye, peritoneum and dermis, especially around the neural tube and among the myotomes. It appears in melanophores at about stage 31, which seems to be earlier than the time stated by Stevens (1954). Between these stages it appears in epidermal cells of the tail and considerable quantities are discharged into the lumen of the gut and that of the brain. Three days' treatment with phenylthiourea beginning at stage 25 markedly reduces the pigmentation of eye, dermis and peritoneum, but does not diminish the pigment in the brain and in the epithelium of the gut.

Between stages 36 and 40, pigment appears in the meninges, in the connective tissue between the muscle fibers of the myotomes and, in traces, in the liver. Of this recent pigment, treatment with phenylthiourea beginning at stage 25 suppresses only that in the meninges. Despite the extended treatment with phenylthiourea pigmentation increases in the gut and central nervous system.

Between stages 40 and 44 in larvae treated for 9 days from stage 26, the drug continues to exert its former marked effect on the eye, peritoneum, olfactory sacs and meninges, but no consistent effect is produced by it on the pigment in the gut, brain or in the connective tissue associated with the tail muscles.

After 14 days' treatment, pigmentation of the eye and dermis is drastically reduced and that of the peritoneum disappears. At stage 46, after 29 days' treatment, no pigment remains in the dermis and only traces in the eye; that in the lining of the gut is somewhat reduced, but that in the cerebral ventricles is unaffected.

A decline in effectiveness appears as development advances to stage 48. Thus, 9 days' treatment, beginning at stage 39, produces no effect, but 30 days' treatment, beginning at stage 42 causes clear loss of pigmentation in the peritoneum, eye, meninges, walls of the blood vessels and in the olfactory organ. The effect on the eye is but slight and in the dermis though pigmentation over the myotomes is still diminished by the drug, that around the neural tube is not. Treatment begun at stage 45 and continued for 26 days produces similar effects.

That pigment is still being formed in these regions between these stages may be shown in a striking manner by suppressing pigmentation with phenylthiourea and then allowing it to return on cessation of treatment. The clearest demonstration is obtained by treating a larva for 29 days beginning at stage 28, when all pigmentation, except that in the epithelium of the gut and in the cerebral ventricles, is lost. After a further 9 days in water without phenylthiourea, by which time the larva is at stage 46, substantial repigmentation of the eye and peritoneum occurs, but rather unexpectedly, little pigment is redeveloped in the dermis and none in the meninges. However, pigmentation of these structures is completely restored in 7 days, following a shorter (14 days) treatment with phenylthiourea beginning at the same stage.

It is noteworthy that phenylthiourea, even after 30 or more days, exerts no consistently clear effect on the melanin in the lining epithelium of the gut, on the small amounts in the liver, or on the considerable quantities that still appear in the cerebral ventricles, though there is some indication of increased amounts of pigment in these situations after treatment. This is substantiated by the experiments in which pigmentation was allowed to redevelop after treatment. Pigmentation in the gut and cerebral ventricles showed a tendency to decrease on cessation of treatment.

By stage 49, the effectiveness of phenylthiourea is drastically reduced. Though an inhibiting effect on the pigmentation of the meninges could sometimes be detected after 23 days' treatment, in other cases no effect of the drug was discernible, even after 52 days.

To the extent that they show a limited period of particularly active pigment formation, these observations agree with those of Sims (1961), who found that phenylthiourea ceased to affect normal melanogenesis at about stage 46. Nevertheless, we find that pigment continues to form beyond this stage.

Although the increase in pigmentation was not followed stage by stage beyond this point, examination of sections of metamorphosing larvae (stage 64) showed that pigment continues to accumulate up to this time, in the dermis, especially dorsally, in the walls of the major blood vessels and in some of the minor ones in the hind limbs, in the lungs and in the membranous labyrinth. A notable increase in pigment is seen in the liver and pancreas. Darkening of the epidermis deserves comment; it is mostly due to extensive penetration of processes from dermal melanophores, but scattered granules also appear, some of which may be in the epidermal cells. At this stage, melanin has completely disappeared from the gut lining and cerebral ventricles.

The effect of phenylthiourea on the pattern of melanogenesis revealed above, not only defines a period of rapid and extensive melanization, but also focusses a clear and significant distinction between areas in which pigmentation is consistently reduced by the substance and those in which it is not. Moreover, in the latter areas there is a hint of the opposite effect occurring, which is difficult to reveal in the absence of any adequate quantitative means of assessing the pigmentation, because the effect is insufficiently large. Accordingly, the effect of phenylthiourea was examined in *Rana pipiens* and *Rana temporaria* during this period of active melanogenesis. Larvae at about the stage of hatching were subjected to phenylthiourea for 19 days in the former species and for 11 days in the latter. The material available was insufficient to permit detailed comparison and formulating general conclusions. Nevertheless, we may note that in the series of sections examined, the areas in which pigmentation was diminished were the same as in *Xenopus* and that the suppressive effects of phenylthiourea were more striking in the dermis, and, in the case of *Rana pipiens,* also in the blood vessels. It is in areas which in *Xenopus* were largely unaffected by phenylthiourea that the most evident differences appear. Thus, the amount of pigment in the liver, lining of the gut, and in the connective tissue and myoblasts associated with the myotomes clearly increased after treatment with the substance. On the other hand, the amount appearing in the cerebral ventricles was unaltered. The increase of the pigment in the myotomes is striking in *Rana pipiens,* where much melanin appears in irregular masses inside the muscle fibers as well as in the surrounding tissue after treatment (Figs. 12 and 13).

An equally significant difference appears in the kidneys where appreciable amounts of melanin appear in the walls of the pronephric tubules; moreover the amount is greatly increased after phenylthiourea (Fig. 5). The same applies, though to a lesser extent, in the mesonephric tubules.

Discussion

In general, melanin has been endowed with an adaptive significance, because it conceals (Kettlewell, 1958), or screens against nocous radiation (Fitzpatrick, 1965; Garn, 1964; Millott, 1954), or transduces energy (Verne, 1926). In some cases its function has been clearly shown, for example, by Kettlewell or by Fitzpatrick, and in others the functions proposed are at least comprehensible when the pigment occurs in appropriate situations, but its adaptive significance is less tangible when the pigment is deep-seated. In such cases it may be excretory (Verne, 1926), although some reluctance to accept the notion has been voiced (Fox and Vevers, 1960).

In the larvae of *Ambystoma mexicanum, Rana temporaria* and *Rana pipiens,* melanin is indeed ubiquitous, but the precise disposition of the pigment in certain situations, such as the epithelium of the gut, the wall of the brain and in the kidney tubules, together with the unmistakable signs of being discharged into their corresponding cavities, strongly suggest it is being excreted, sometimes in considerable quantities. It should not be assumed, however, that the pigment is excreted in the same sites as those in which it was formed, and in this connection the different responses to phenylthiourea might be explained. Thus, the sites at which pigmentation is lessened by the substance may be the areas of pigment formation and accumulation, whereas those in which it is not may be the sites of its excretion. Histological appearances, the disposition of pigment in areas for which it is difficult to conjecture an adaptive significance, signs of the existence of tyrosinase only in maturing melanophores (Sims, 1962) and indications of the capacity of such cells, in these and in other animals, to hand on their melanin to other cells (Fitzpatrick, 1965), all fit with such a notion. Moreover, the temporal pattern in *Xenopus,* described above, is similarly suggestive, for when the larvae begin to form their own pigment, between stages 25 and 31, excretion in such areas as the gut and brain is rapidly increased and persists in these areas for so long as active synthesis persists (judged by the effectiveness of the suppressive action of phenylthiourea), until about stage 49. Although discharge of pigment paradoxically precedes signs of its formation in early larvae, such pigment is unaffected by phenylthiourea and appears to be egg pigment carried over from the mother.

It is noteworthy that the two processes are not completely in phase, because melanogenesis begins to decline between stages 40 and 44, whereas significant quantities appear in the gut until stages 49 to 51, and in the brain until stage 52. Although these stages roughly mark the end of a notably active phase of pigment formation, production, accumulation and elimination of melanin continue in other sites at least up to the time of metamorphosis. As regards the liver, it is pertinent to recall the remarks of Foxon (1964) concerning the ingestion of melanin by leucocytes and macrophages, as well as those of Niu and Twitty (1950), who describe the transfer of pigment by "melanophages" from the skin of *Triturus* to the liver at metamorphosis.

Such an interpretation of the histological picture and the effect of phenylthiourea implies that active turnover of pigment occurs. This agrees with our findings in *Eleutherodactylus* (Millott and Lynn, 1954) but not with those of Sims (1961) in *Xenopus.* Our findings in *Xenopus* are reinforced by those in the other species briefly examined, where more melanin is formed, some of which is laid down in structures where little, if any, is found in *Xenopus,* notably in the epidermis.

Its presence inside the epidermal cells as well as in penetrating melanophores recalls the statements of Ehrmann (1885) concerning *Hyla* and *Salamandra* and Stearner (1946) concerning *Ambystoma* and *Triturus.* Moreover, the appearances reported in *Rana pipiens* (Fig. 1) strongly suggest that melanin is transferred to epidermal cells from melanophores, though we have no evidence to show that this is done in the precise way indicated by Stearner. If, indeed, such transfer occurs, the situation in Amphibia is brought more closely into line with that in birds (Stearner, 1946) and mammals (Fitzpatrick, 1965).

It is significant that the suppression of pigmentation is not the sole effect of phenylthiourea. There are indications, slight in *Xenopus,* but more substantial in the other species we have studied, of the opposite effect. The fact that treatment with phenylthiourea increases the amount of melanin in areas such as the gut and kidney, where there are clear signs of pigment being excreted, constitutes a strong hint that existing ideas about the action of the substance are, to say the least, inadequate. The amount of material available, and the number of experiments that could be performed, forbid more positive and specific assertions being made, but possible effects of phenylthiourea on storage, accumulation, or excretion of the pigment merit further examination.

We are grateful for the advice of Dr. A. S. G. Curtis concerning the administration of "Pregnyl" to *Xenopus,* and for the skilled assistance of Mr. Z. Podhorodecki in photography.

SUMMARY

1. The widely distributed black or brown pigments in the larvae of *Xenopus laevis, Rana pipiens* and *Rana temporaria* were examined histochemically and found to behave like melanin.

2. The distribution of this pigment in *Xenopus* larvae up to the time of metamorphosis is described and compared with its distribution at certain stages in *Rana pipiens* and *Rana temporaria.*

3. The development of pigmentation is studied in *Xenopus* up to the time of metamorphosis.

4. The effect of 0.01% phenylthiourea on pigmentation in *Xenopus* is studied up to the time of metamorphosis. Its effect is compared with that observed at certain stages in *Rana pipiens* and *Rana temporaria*. In some areas of the larvae, pigmentation is reduced by phenylthiourea, in others it is unaffected or even increased by it.

5. The significance of the differing responses to phenylthiourea is discussed in relation to the development and disposition of pigment, histological evidence of the excretion of melanin and the problem of pigment turnover.

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PHYLOGENY AND THE DISTRIBUTION OF CREATINE IN INVERTEBRATES[1]

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Studies of the distribution of phosphagens have been interpreted in phylogenetic terms since their inception. Kutscher and Ackermann (1926) suggested that the terms "creatinate" and "acreatinate" might appropriately replace "vertebrate" and "invertebrate." This reflected the opinion that creatine was not merely the characteristic phosphagen of vertebrates but was diagnostic of the sub-phylum.

Most biologists are familiar with the phylogenetic implications drawn from the data of Needham *et al.* (1932) and Baldwin and Needham (1937). These workers concluded that creatine occurred as a phosphagen in some echinoids and ophiuroids as well as in some hemichordates. This, taken together with the supposed diagnostic significance of this compound, was widely accepted as providing biochemical support for the echinoderm ancestry of the vertebrates.

More recent work has led to a reinterpretation of these data so that this biochemical support of phylogenetic orthodoxy now seems less cogent. Yudkin (1954) expressed reservations on this score in his paper on phosphorylation in echinoderms. Creatine has been definitely identified in several annelids and has also been found in sponges and coelenterates (Roche *et al.*, 1957). These authors conclude that this scattered distribution precludes assigning any phylogenetic meaning to the presence of creatine. This view is supported by Ennor and Morrison (1958) in their review and by Kerkut (1960). After reviewing the literature, the latter author states (page 127) that "there is certainly no simple cleavage of the animal kingdom into vertebrates with CP [creatine phosphate] and invertebrates with AP [arginine phosphate]. Instead it is clear that both CP and AP are found throughout the invertebrates." Further (page 127) "one cannot base any pylogenetic speculation on the occurrence of CP or AP since related genera within a class can differ widely in their phosphagens." Finally, he points out that pylogenetic interpretations are also complicated by the recent isolation of several additional substituted guanidines which apparently serve as phosphagens in some invertebrates. These remarks express what seems to be a consensus of opinion, although Baldwin (1963) continues to allude to the distribution of creatine as phylogenetically meaningful.

Despite numerous contributions and even more numerous allusions to this subject, it is clear that the criteria for assigning evolutionary significance to such data have not received as much attention as they deserve. This is in sharp contrast to the care with which the biochemical procedures have been scrutinized. It is also in marked contrast to the care with which the foundations of comparative biochemistry have been discussed in other contexts. Wald (1963) has pointed out in a

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discussion of the distribution of visual pigments that great care must be exercised in attributing phylogenetic meaning to the occurrence of the same molecule in various organisms. He concludes that the presence of retinene in three major phyla as a key constituent of visual pigments is evidence for the unique properties of this molecule rather than evidence for any relation between molluscs, arthropods and vertebrates.

The immediate aim of the present work is to provide quantitative information concerning the occurrence of creatine and guanidinoacetic acid in selected invertebrates. In addition to identifying and estimating these substituted guanidines, we have assayed for transamidinase activity in homogenates of these organisms. Transamidinase is a key enzyme in the apparently unique synthesis pathway of creatine which is found in mammals. It funtions to transfer the amidine group from arginine or another donor to glycine. The guanidinoacetic acid produced by this reaction is then methylated to form creatine.

It became apparent in the course of these observations that the marine invertebrates which were employed were capable of accumulating creatine from extremely dilute solutions. In a number of cases, these same organisms had been reported to be capable of removing glycine from dilute solution in the ambient sea water (Stephens and Schinske, 1961; Stephens, 1963, 1964). This was verified and the observations extended to include uptake of arginine. The capacity of the organisms to take up these compounds made possible additional observations of interest with respect to the synthetic capacities of these forms.

It was hoped that providing quantitative information concerning the occurrence of creatine, supplemented by information concerning the activity of this key enzyme in its synthesis, might add to our understanding of the significance, if any, of the distribution of substituted guanidine compounds among the invertebrates.

MATERIALS AND METHODS

Organisms were obtained from several sources and shipped to Minnesota where most of this work was carried on. *Glycera dibranchiata* was purchased from "The Maine Bait Company" and presumably had been collected in the vicinity of Newcastle, Maine. *Mercenaria mercenaria* was supplied by a local wholesale fish dealer in Minneapolis. Other forms were obtained from the Marine Biological Laboratory, Woods Hole. Animals were held briefly in moist *Fucus* or in artificial sea water until extracts were prepared or other observation procedures undertaken. Animals incubated with C^{14}-labelled compounds were exposed to dilute solutions of the material in question at 20° C. for the periods indicated.

Creatine was determined according to the procedure described by Van Pilsum *et al.* (1956) and creatine phosphate as indicated by Van Pilsum (1957). The analysis depends on quantitative conversion of creatine to methylguanidine. This procedure gives unequivocal information concerning the presence and amount of creatine, provided the presence of argininosuccinic acid is excluded, as described by Van Pilsum and Halberg (1962). This was done whenever creatine was found. Analyses were carried out using 10% homogenates in 0.6 N perchloric acid which were prepared, neutralized with KOH, and filtered, all at 0° C. One aliquot was analyzed for total creatine. Another was passed through a Dowex-50 column retaining the creatine and passing creatine phosphate. After conversion of creatine to methylguanidine, this compound was measured colorimetrically by a modified Sakuguchi reaction. The limit of sensitivity of the creatine determination as described is approximately 5 mg. per 100 gm. tissue. Creatine was added to samples of all homogenates as a control procedure. Recovery was quantitative.

Whenever creatine was detected by this procedure, an aliquot of the protein-free extract described above was evaporated to dryness and extracted with petroleum ether to remove lipid. The extracted residue was dissolved in water and desalted by electrodialysis. The material was concentrated and spotted on filter-paper for chromatography. Three different solvent systems were routinely used. They were *n*-butanol-glacial acetic acid-water, 120:30:50; *n*-butanol-pyridine-water, 65:65:65; and isopropyl alcohol-ammonia-water 200:10:20 (Smith, 1960). Location reagents were prepared according to Smith (1960). Sakaguchi reagent was used for locating guanidinoacetic acid and methylguanidine, and diacetyl reagent for creatine.

Transamidinase was determined by the procedure described by Van Pilsum *et al.* (1957) and by a more sensitive modification of this technique (Van Pilsum *et al.,* 1963). Aliquots of a water homogenate (5% by weight) of the organism concerned were incubated with equimolar amounts of canavanine and glycine at pH 7.4. Canavanine is an analogue of arginine. It was used because it is a more effective amidine-group donor than arginine. In most cases, when transamidinase activity was found, the assay was repeated using arginine as a donor. Before and after incubation protein-free filtrates were prepared, using barium hydroxide and zinc sulfate. The guanidinoacetic acid formed was measured in this filtrate, using a modified Sakuguchi reaction. Whenever transamidinase activity was found, aliquots of this filtrate were extracted with petroleum ether and desalted and chromatographed using the solvent systems described above. The limit of sensitivity of this procedure is approximately 50 micrograms of guanidinoacetic acid formed per gram wet weight in six hours of incubation.

We were concerned to report our negative results and attach some significance to them. In order to reduce the probability that negative results for transamidinase activity were the result of some inhibitor in our homogenates, rat kidney homogenate was added as a routine control procedure. The transamidinase activity was recovered in all cases. To check the possibility that guanidinoacetic acid was being formed and entering some further reaction sequence, an aliquot of homogenate was incubated with added guanidinoacetic acid. In all cases where negative results for transamidinase were obtained, the added guanidinoacetic acid was recovered quantitatively. In certain cases the incubation mixtures were analyzed for creatine as well, to exclude the possibility of its synthesis.

Uniformly labelled glycine-C^{14} was supplied to the organisms indicated as dilute solutions in sea water. Creatine-methyl-C^{14} was employed for most of these observations although some initial observations were made using creatine labelled in the carboxyl position. Radioactivity of the ambient medium and appropriate extracts was determined using a thin-window Geiger tube. Data presented are corrected for background and sample thickness. Chromatograms of radioactive compounds using three different solvent pairs were scanned and compared with chromatograms of authentic labelled material. Creatine was also located using the diacetyl reagent in chromatograms of radioactive material.

RESULTS

The organisms used can be divided into three groups based on data obtained concerning the presence of creatine and guanidinoacetic acid and the presence of transamidinase activity. In one group of the invertebrates tested, neither of the substituted guanidines was found nor could transamidinase activity be detected. Detectable levels of transamidinase activity and guanidinoacetic acid were present in a second group but no creatine could be demonstrated. Finally, the third group possessed creatine but no guanidinoacetic acid and no transamidinase activity could be detected. These relations are indicated in Table I. It should be indicated that no selection has been exercised; all animals examined fall into one or another of these three groups.

TABLE I

Transamidinase activity is expressed as micrograms of guanidinoacetic acid formed per gram of tissue after six hours of incubation. A dash means less than 50 micrograms. The column labelled guanidinoacetic acid refers to the presence of a spot with the R_f value of this compound on a paper chromatogram. A protein-free extract of the organism was used as described in the text. Creatine content is expressed as milligrams per 100 grams wet weight of the organism. A dash means less than 5 mg./100 gm.

Species (phylum)	Transamidinase activity	Guanidinoacetic acid	Creatine
Glycera dibranchiata (Annelida)	—	—	500
Diopatra cuprea (Annelida)	—	—	100–200
Saccoglossus kowalevski (Hemichordata)	—	—	30
Styela carnea (Chordata)	—	—	15–35
Metridium dianthus (Cnidaria)	100	+	—
Nereis virens (Annelida)	250	+	—
Lepidonotus squamatus (Annelida)	900	+	—
Mercenaria mercenaria (Mollusca)	300	+	—
Amphitrite ornata (Annelida)	—	—	—
Golfingia gouldii (Sipunculoidea)	—	—	—
Spisula solidissima (Mollusca)	—	—	—
Thyone briareus (Echinodermata)	—	—	—
Leptopsynapta inhaerens (Echinodermata)	—	—	—
Ciona intestinalis (Chordata)	—	—	—
Amaroucium constellatum (Chordata)	—	—	—

Creatine phosphate was demonstrated qualitatively in the four animals which showed detectable creatine. In *Glycera*, 285 mg. per hundred grams wet weight, or 57% of its creatine, was in the form of creatine phosphate. Since it was somewhat unexpected to find that the organisms which possessed creatine showed no transamidinase, additional observations were undertaken. Efforts to identify creatine in protein-free extracts of *Nereis* and *Mercenaria* by chromatography were negative. Five per cent homogenates of *Glycera, Diopatra, Styela* and *Saccoglossus* were incubated with canavanine and glycine at pH 7.4 and examined for possible creatine formation both colorimetrically and chromatographically. No creatine synthesis could be demonstrated.

Saccoglossus and *Glycera* were exposed to dilute solutions of uniformly labelled arginine-C^{14} and glycine-C^{14} for periods of one to four hours. They were then maintained for 24 to 96 hours in sea water and sacrificed. A perchloric acid extract of the animals treated in this way showed considerable radioactivity as a result of the accumulation of these amino acids from the surrounding medium.

The extracts were desalted and defatted, chromatographed, and passed through the chromatogram scanner. Clear peaks of radioactivity were present at the R_f values of authentic samples of arginine or glycine, respectively. Creatine could be located using diacetyl reagent. No radioactivity was found in any experiment of this sort associated with the creatine. Thus, the organisms could not be demonstrated to synthesize creatine from appropriate precursors by this approach, during periods as long as four days.

Several of these species were also examined with respect to their ability to accumulate creatine from dilute solution in the surrounding sea water. *Saccoglossus, Glycera, Nereis* and *Mercenaria* all exhibited this ability. Details will be presented for *Glycera*.

When individual bloodworms were exposed to creatine-C^{14} labelled in the carboxyl position at concentrations as low as 10^{-7} moles per liter of sea water, the labelled material was accumulated. Chromatograms showed radioactivity at the same R_f as that exhibited by authentic creatine. Creatine labelled in the methyl position behaved in the same way.

The relation between weight and rate of uptake was exponential (*cf.* Stephens, 1963). For 24 worms ranging in weight between 2 grams and 11 grams, a plot of the log of wet weight *versus* the log of rate of uptake gave a reasonable fit to a straight line. The least squares regression line had a slope of 0.49 ± 0.05. The correlation coefficient was 0.907. Hence, rate of uptake was taken to be linearly related to the square root of wet weight. This relation was used to reduce subsequent data to a form which permitted comparison among animals of different weight. An effort was made to minimize size variations.

It seems likely that accumulation occurred directly across the body wall since rate of uptake by individuals ligated at the head and tail was not significantly different from that shown by normal individuals. The rates in arbitrary units were 1.26 ± 0.29 ($N = 6$) and 1.41 ± 0.35 ($N = 6$), respectively.

Rate of accumulation was measured at concentrations ranging from 2×10^{-5} moles per liter to 10^{-3} moles per liter. The reciprocal of the measured rate was plotted against the reciprocal of ambient concentration. The resulting points fell along a straight line, permitting estimation of maximum rate of uptake and the concentration at which uptake is half maximal. The procedure is analogous to estimation of $V_{\max}$ and K_m by using a Lineweaver-Burk plot. Of course no implication that the process is enzymatic is intended. However, the fit of the data to a straight line suggests an adsorptive rate-limiting step at higher ambient concentrations. $V_{\max}$ for creatine uptake is 1.05 ± 0.15 micromoles per hour per gram for worms weighing 7.35 ± 0.59 grams ($N = 12$). K_m is approximately 2.0×10^{-4} moles per liter of creatine.

Creatine was not readily lost to the environment by these worms nor was it easily exchanged. After 96 hours, radioactive creatine labelled with C^{14} in either the methyl or the carboxyl group could still be demonstrated. Chromatograms verified that radioactivity was still in the form of creatine. Insufficient quantitative data are available to calculate a rate of loss of labelled creatine but it is apparently quite slow since no marked decline in activity was found at the end of this period. A group of 24 animals was exposed to creatine-C^{14} for 60 minutes, rinsed in sea water for five minutes, and placed in 50 ml. of sea water containing 10^{-4} moles per liter of unlabelled creatine No radioactivity above background was found at the end of two hours. Extracts of several worms selected at random ranged between 1000 and 2000 cpm. per gram. Hence, exchange of creatine with the environment appears to proceed extremely slowly.

Less data are available for *Saccoglossus,* but it removed creatine from dilute solution rapidly. *Nereis* and *Mercenaria* also showed this capacity. Some data are available which suggest that creatine-C^{14} was lost or degraded in *Nereis* over a period of 48 to 96 hours after its accumulation. Extracts made 48 hours after exposure to radioactive creatine showed only about one-third of the activity exhibited by extracts made immediately after exposure. Only four specimens were measured but this result contrasts with the failure to demonstrate depletion in *Glycera.* Chromatography established that radioactivity still resided in creatine when carboxyl-labelled or methyl-labelled material was used. However, there was an additional radioactive spot in the case of the creatine-methyl-C^{14}. This has not been firmly identified, but suggests degradation of creatine before its elimination.

An effort to detect creatine in sea water samples collected in regions where *Glycera* is present was unsuccessful. This implies that concentrations are less than 1 mg. per liter if creatine is present.

Discussion

The distribution of creatine, guanidinoacetic acid, and transamidinase activity found in the present investigation is particularly intriguing in those invertebrates which exhibit creatine: none has any demonstrable transamidinase. Several antecedent possibilities can be indicated. It may be that transamidinase is present but activities are too low to be demonstrated by the present procedure. Alternatively, creatine may be synthesized by some pathway which does not involve the transfer of the amidine group to glycine in the usual fashion. As yet another possibility, one might speculate that demonstrating invertebrate transamidinase activity requires conditions which are not provided in these experiments. None of these possibilities seems likely. It must be borne in mind that no selection of cases has occurred. All the organisms which show creatine fail to show transamidinase activity. It would be unusual to pick four phylogenetically scattered organisms, all of which just happen to have very low levels of transamidinase despite having considerable amounts of creatine in their tissues. The uniform presence of transamidinase activity in homogenates of organisms having guanidinoacetic acid indicates that there is no widespread set of highly specific requirements for demonstration of transamidinase in invertebrates. Finally, the hypothesis that some new pathway of synthesis is involved receives no support from the attempts to produce labelled or unlabelled creatine *in vivo.* This is not to say that these possibilities can be totally discarded. However, none of them receives any support from our observations and none of them has very high antecedent probability.

Another explanation can be advanced. The invertebrates which show creatine may acquire it from some exogenous source. This hypothesis has some obvious attractions and is supported by our data. If creatine were acquired by marine invertebrates as part of their food or by accumulation from dilute solution in their environment, then a more satisfactory account of the extremely scattered distribution of this compound among invertebrates could be offered. Organisms such as *Glycera* might be thought of as employing creatine as a phosphagen in an adventitious fashion. Presumably, creatine might be available to any organism feeding on certain diets or adapted to accumulate it. However, only some organisms would develop the phosphoryl transfer enzyme necessary to employ it as a phosphagen. If this were the case, neither the presence of creatine as an isolated compound nor its use as a phosphagen would have phylogenetic significance.

In support of this possibility, it can be noted that one would expect to find no transamidinase associated with creatine since by hypothesis, it would be acquired exogenously rather than synthesized. One would also expect to find other organisms in the same general area which were capable of acquiring creatine but which eliminated it without using it as a phosphagen. This appears to be the case in *Nereis* and *Mercenaria*. Finally, this permits some reconciliation of earlier views concerning the phylogenetic significance of creatine distribution with our more recent information concerning the occurrence of this compound among invertebrates. It seems very reasonable to demand that if a compound is to carry the phylogenetic freight that has been borne by creatine in past decades, it should meet more rigorous standards than are currently stipulated. In particular, one should ask for a demonstration of some continuity in the pattern of synthesis and degradation of the material in question. This might be compared to the demand that morphologically comparable units or homologues must exhibit the same larger pattern and not merely identity of structure in some self-contained sense. Outstanding examples come to mind in the discussion of evolution and protein structure (Margoliash, 1963).

No creatine was found in the habitat of these organisms. It is true that the search was casual. It is also true that one can think of potential sources. Creatine is an excretory product in some marine fishes (*cf.* Prosser and Brown, 1961). It has also been reported as present in soils from various locations (Schreiner and Skinner, 1912) and as a constituent of a number of plants (Guggenheim, 1958). It is not *prima facie* unreasonable that small amounts of creatine might be present in solution in the sea water of estuarine locations nor is it unreasonable that creatine might be present in detritus or some other dietary constituents. However, this remains to be demonstrated.

The present observations indicate the need for reexamination of the distribution of creatine and transamidinase to provide the data necessary for a more sophisticated approach to the possible phylogenetic significance of phosphagen distribution. We support the position that the distribution of creatine among invertebrates as an isolated observation probably has no phylogenetic implications. However, this should not be taken as a denial of the possibility of biochemical support for phylogenetic conclusions based on studies of phosphagen distribution. One should rather demand that conclusions concerning phylogeny be based on more adequate criteria defining homology at the molecular level. Put in another way, our present information permits interpreting the presence of creatine in the invertebrates we have examined as adventitious and, therefore, irrelevant to a phylogeny which may well be reflected in the distribution of creatine and the supporting biochemical matrix in which it is embedded in vertebrates.

SUMMARY

1. Fifteen species of invertebrates from seven phyla were examined for the presence of creatine, guanidinoacetic acid, and transamidinase.

2. None of the animals which possessed creatine had detectable levels of transamidinase. All animals which possessed guanidinoacetic acid also possessed transamidinase activity. A third group of invertebrates was formed of those species in which neither of the guanidine compounds nor transamidinase could be demonstrated.

3. Attempts using various procedures to obtain synthesis of creatine from appropriate precursors in those organisms in which it was found were uniformly negative.

4. Accumulation of creatine from very dilute solution in the ambient medium was demonstrated for all of the invertebrates in which creatine was found.

5. In *Glycera* and *Saccoglossus,* both of which possess creatine, creatine-C^{14} obtained from the ambient medium persists as creatine for at least 96 hours.

6. Other organisms which do not normally possess creatine also showed the ability to accumulate it from dilute solution. Evidence is presented that such animals eliminate creatine and may degrade it in the process.

7. These findings are discussed and their possible significance for phylogeny considered. It is suggested that the creatine found in the invertebrates examined here may be exogenous in origin. Thus, its presence may be adventitious and its use as phosphagen opportunistic.

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INTERACTIONS OF DIURNAL AND TIDAL RHYTHMS IN THE FIDDLER CRAB, UCA PUGNAX[1]

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One of the most nearly universal characteristics of circadian rhythms is the susceptibility to phase-shifting by light. It has been shown to be effective not only for rhythms which under natural conditions are synchronized with a solar-day cycle but also for some which are tidal in nature. Where a diurnal and a tidal rhythm are simultaneously present it is of interest to determine the characteristics of both rhythms with respect to their relative susceptibility to shifting by light. A species known to possess both of these rhythms is the fiddler crab, *Uca pugnax*. Rhythms of both solar-day and tidal periods have been described for color change, oxygen consumption and locomotor activity. The susceptibility of the daily rhythm of color change to shifting by light has been extensively investigated and the characteristics are well established (Webb, 1950). It has also been found that a light-induced shift of the diurnal rhythm is accompanied by a shift of the tidal rhythm, the shifts being of approximately the same magnitude (Brown *et al.*, 1953). This situation suggests either a coupling of some kind between the two rhythms or very similar sensitivities and reactions. Since the expression of the two rhythms requires a continuously changing phase relationship between them it is obvious any coupling that may exist is not total.

Less extensive investigations of the characteristics of the rhythms of locomotor activity have been reported. The crabs show peaks of locomotor activity near the times of low tide and these phase relations are established by some factor associated with the local tides. Thus, animals from two beaches in the same general area but with tide times five hours different show tidal rhythms out of phase with each other by about five hours (Bennett, Shriner and Brown, 1957). There is no evidence that, under such circumstances, the diurnal rhythms are out of phase with each other.

The purpose of this report is to describe the tidal rhythm of locomotor activity of *Uca pugnax* under a variety of light regimes, and some of the characteristics of the apparent linkage between the diurnal and tidal rhythms.

EXPERIMENTAL METHODS

The locomotor activity of male *Uca pugnax*, collected at Chappaquoit Beach, Cape Cod, was recorded by the method described by Bennett, Shriner and Brown in 1957. In some cases the animals were placed in the recording apparatus on the day of collection and the recording carried out under conditions of constant light at an intensity of less than 1 f.c. In some cases recording, under the same conditions, was begun on the day after collection. Since there was no discernible difference between results obtained in the two cases the data have been combined. A total of 70 days of data obtained under these recording conditions is available from two different years, 1961 and 1964, representing about 12 different collections. The times of low tide on the days of collection were distributed through the daylight hours.

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Activity was also recorded under conditions of natural illumination with the animals protected from direct sunlight by a drawn window shade. Two simultaneous series of records, each for a 12-day period, were obtained during September, 1964. The animals were placed in the activity chambers the day after collection. Three simultaneous series, each for a 28-day period, were recorded from June 28 to July 26, 1962. These animals were exposed to an artificial schedule (LD, 12:12; L 50 f.c.) for five days prior to recording activity which was carried out under LL conditions, (L 1 f.c.). Three types of LD cycles were used: (1) light on 6 AM to 6 PM; (2) light on 2 PM to 2 AM; and (3) light on 10 PM to 10 AM. At the end of 15 days the animals in the activity chambers were replaced by other animals that had been pretreated in the same ways.

A total of 40 days of recordings was obtained in 1964 under conditions of constant light of approximately 1 f.c. but with a supplemental lighting of two hours at 55 f.c. The supplemental lighting provided a 24.75-hour cycle since the light period began 45 minutes later each day.

Finally a series of 11 days of data was obtained in 1964 with conditions of constant light at 1 f.c. and with a 24.75-hour cycle provided by means of a supplemental light of 55 f.c. during four hours of each cycle.

Methods of Analysis

Locomotor activity for each hour of each test period was expressed as per cent of hour active, each such value representing the average of from 3 to 6 animals. The time of occurrence of the highest value for each half-day was then recorded. Next, the times of occurrence of local low tides were recorded for each day for which data were available. Finally, the time of highest activity for each half-day was plotted as a function of the time of nearest low tide. When all the data for a given set of conditions are plotted together in the manner described, and similar plots made for each set of conditions one should be able to answer two questions. (1) Is there a characteristic relation between the time of occurrence of maxima and time of low tide? (2) If such a relationship exists is it influenced by pretreatment or conditions of recording? The method obviously places certain arbitrary limits on the time relations possible. But if the high values are distributed randomly with respect to time of day and time of tide one would obtain a plot with points falling randomly within a diagonal band whose limits are time of low tide plus and minus 6 hours. If, on the other hand, there is a consistent relationship to time of tide, only a portion of this band would be occupied. An advantage of this method of plotting is that if there is a systematic variation of the time relationships occurring throughout the lunar month, such a variation should be evident in the graph. The usual method of obtaining a mean lunar-day curve requires the averaging of values for an unbroken series of data for 15 days or, preferably, for 29 or 30 days, and obviously does not show the nature of any systematic variations within the period for which the curve is obtained.

RESULTS

In Figure 1 are plotted the data for all of the experiments in which there was no pretreatment and recording was under LL conditions. Ordinate values represent time of maximum activity, abscissal values are time of occurrence of low tide. The vast majority of points fall between the time of low tide and six hours before low tide. It is evident, however, that between hours 1 and 5 of the solar day many points fall in the three hours before low tide, while between hours 8 and 14 of the solar day practically all of the points lie between 3 and 6 hours before low tide. In other words, while the tides are progressing from about 6 AM to noon, there is almost no corresponding progression in time of occurrence of activity maxima. There then occurs a rapid progression of the activity maxima so that by

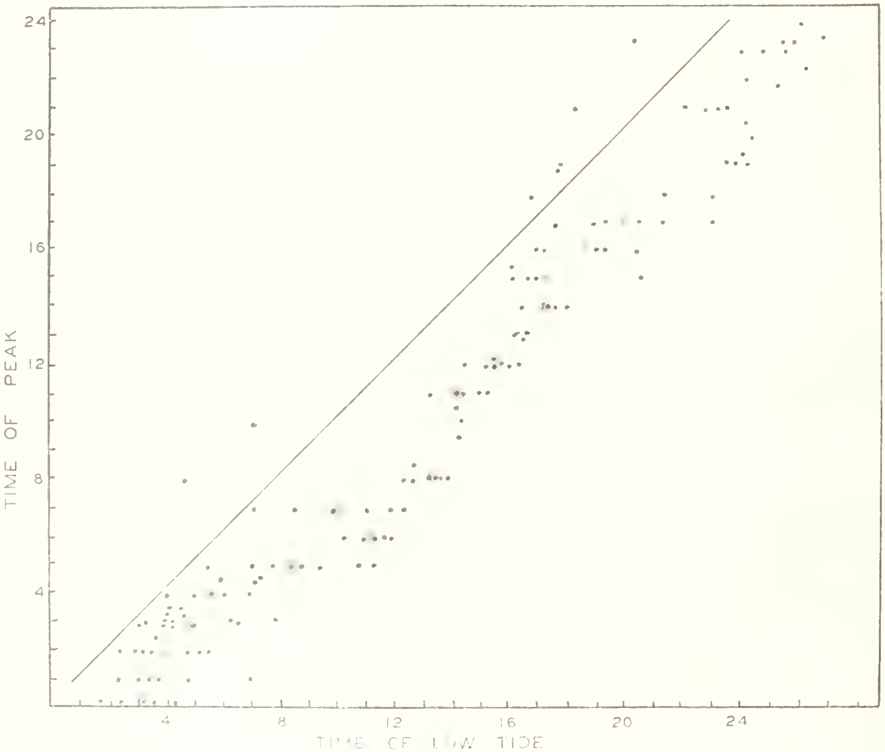

FIGURE 1. Time of day at which peak of locomotor activity occurs (ordinate) in relation to time of day of nearest low tide (abscissa). Recording in constant illumination. The diagonal represents the positions of peaks if they coincided with times of low tides.

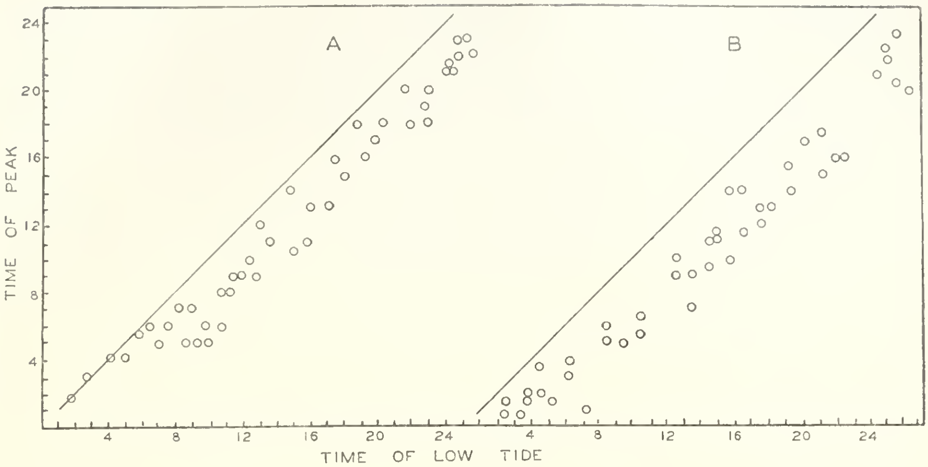

FIGURE 2. A. Time of activity peaks in relation to time of low tides for animals pretreated with light 6 AM to 6 PM and recorded in constant illumination. B. Times of activity peaks for animals without pretreatment and recorded in natural illumination.

the time the tides occur at 17 hours the peaks occur at almost the same time. It should be noted that these variations were not dependent on the length of time the animals had been in the laboratory or in the recorders.

Figures 2A and B show comparable data for animals pretreated by exposure to 5 days of light on from 6 AM to 6 PM (2A) and for animals recorded under conditions of natural illumination (2B). In both cases the majority of points fall in the

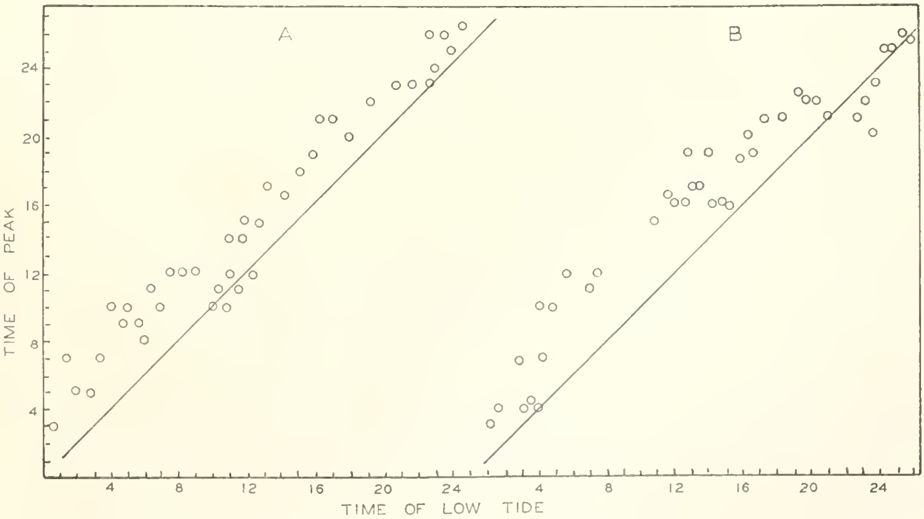

FIGURE 3. A. Time of activity peaks in relation to time of low tide for animals pretreated with light 2 PM to 2 AM and recorded in constant illumination. B. Time of activity peaks for animals pretreated with light 10 PM to 10 AM; recorded in constant illumination.

6 hours preceding low tide. The same tendency of the peaks to remain stationary while the tides progress from 6 AM through the morning hours is seen in Figure 2A as was noted in Figure 1. However, there is no such delay evident in Figure 2B. Thus, the exposure to natural illumination seems to have resulted in a more accurate expression of the tidal rhythm on a day-to-day basis—but not more accurate on the average.

The data obtained from animals pretreated by exposure to 5 days of light from 2 PM to 2 AM are shown in Figure 3A, and those for animals pretreated by exposure to light from 10 PM to 10 AM are seen in Figure 3B. In contrast with any of the preceding cases the majority of these points are seen to fall in the period following the time of low tide. In Figure 3A a delay in progression of the peaks occurs, such that as the time of low tide approaches noon the peaks coincide with it or even precede it; a similar condition seems to occur just before midnight. Similarly in Figure 3B, there is conspicuous delay in progression of the peaks as tides move from about 6 PM to midnight.

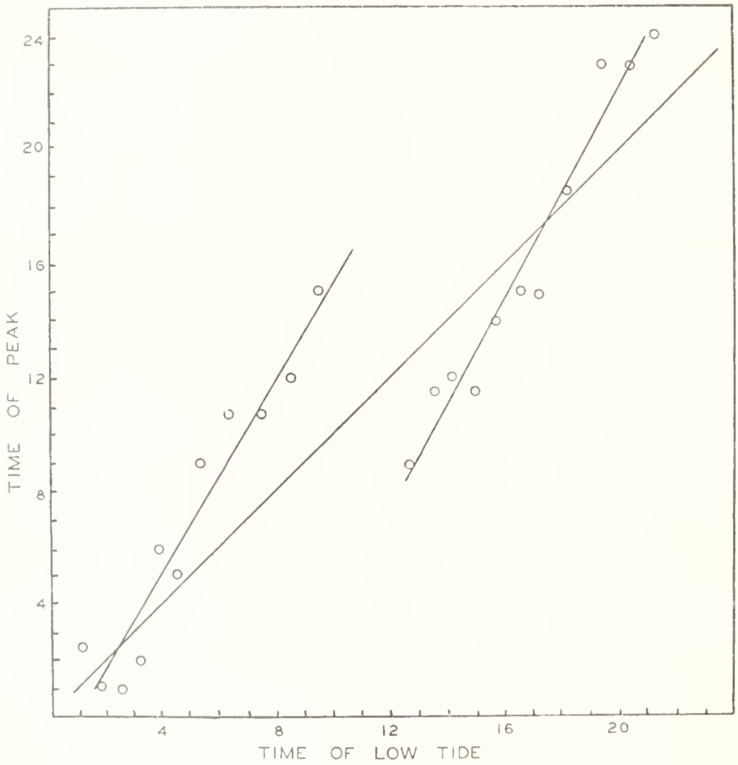

FIGURE 4. Time of activity peaks in relation to low tide for animals exposed during recording to cycle of 24.75 hours by four-hour supplemental light.

The data obtained for animals exposed to a 24.75-hour cycle by supplemental light periods of four hours duration are seen in Figure 4. The points in this case seem not show any systematic variation in rate of progression but rather to progress consistently at a rate greater than that of the tides. The period calculated from the slope of the line drawn through the points is about 25.5 hours.

The data for animals exposed to a 24.75-hour cycle by supplemental light periods of two hours duration are seen in Figure 5. There was less consistency between samples here than for any of the previous experiments. In a few cases the average rate of progression of peaks over a period of 10 or more days was greater than the rate of tidal progression; in most cases the peaks occur within the six-hour period preceding low tide but with no clear pattern within that period.

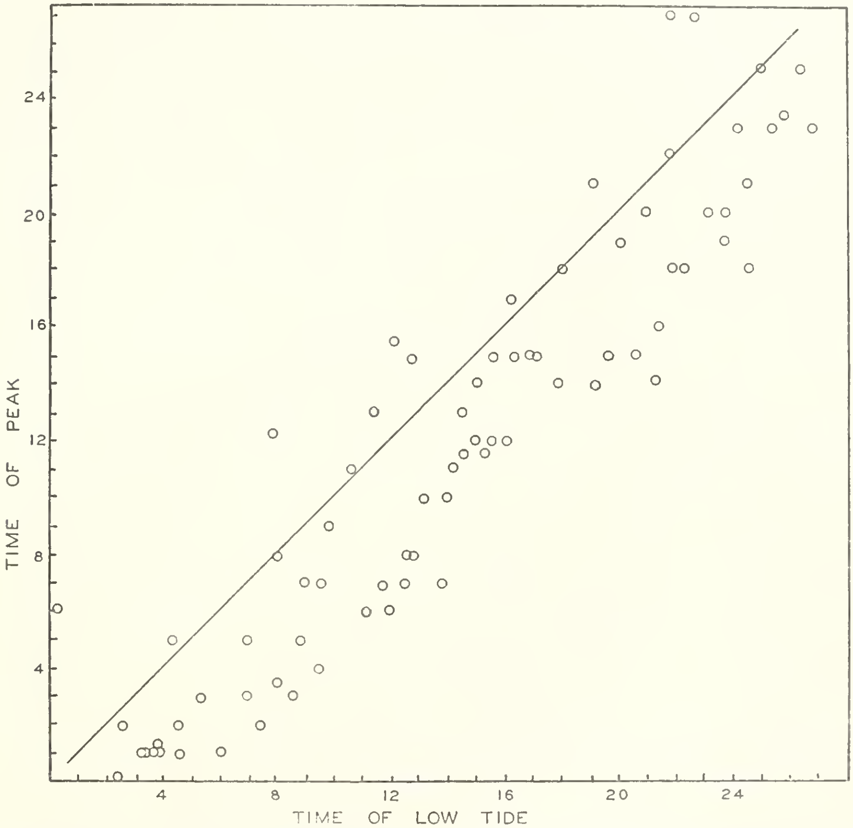

Figure 5. Time of activity peaks in relation to low tide for animals exposed during recording to cycle of 24.75 hours by two-hour supplemental light.

In Figure 6 are presented mean diurnal curves for those experiments for which two successive 15-day periods of data were available. Figure 6B shows the mean curve for animals pretreated by exposure to light from 6 AM to 6 PM for 5 days; ordinate values are per cent of hour active, along the abscissa are plotted hours of the solar day. Figure 6C presents comparable data for animals pretreated by exposure to light from 10 PM to 10 AM; Figure 6D, the data for animals exposed

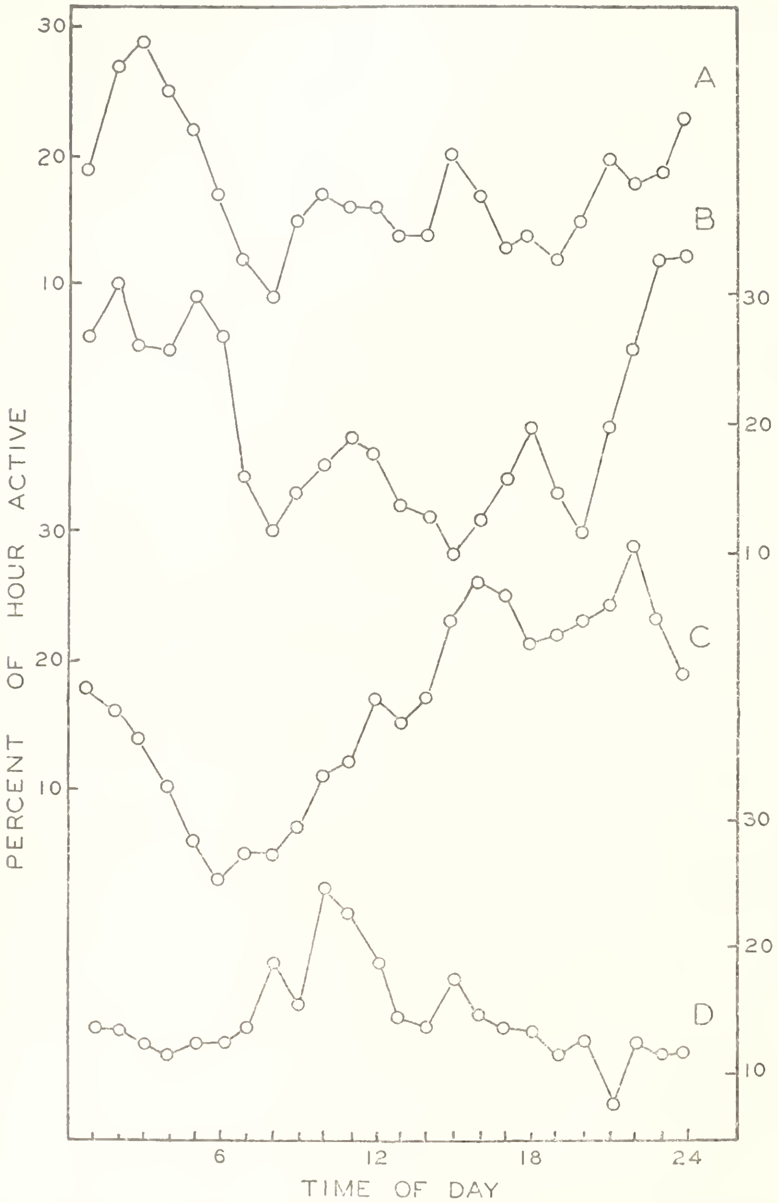

FIGURE 6. Mean diurnal curves: A. For animals without pretreatment. B. For animals pretreated with light 6 AM to 6 PM; C. For animals pretreated with light 10 PM to 10 AM; D. For animals pretreated with light 2 PM to 2 AM. All recorded in constant illumination.

to light from 2 PM to 2 AM; Figure 6A, the data for animals without pretreatment and recording begun within a day of collection. Animals that had experienced an LD cycle essentially in phase with the normal (Fig. 6B) show a high level of activity from about 10 PM to 6 AM. Animals whose artificial dawn was 8 hours early (−120°) show a high level of activity from about 3 PM to midnight (Fig. 6C). Their cycle is thus shifted by 6 to 7 hours. Animals whose artificial dawn was 8 hours late (+120°) show a peak of activity at about 10 AM (Fig. 6D). Using the mid-point of activity seen in Figure 6B as a basis for comparison, one arrives at an estimated shift of 8 hours for the curve in Figure 6D. The phase relations of the diurnal rhythm of the untreated animals (Fig. 6A) seem to be the same as those seen in Figure 6B.

Discussion

The results of these experiments suggest two kinds of relationship between diurnal and tidal rhythms of locomotor activity. One kind of relation is seen for all animals whose activity was recorded under conditions of constant light. Under these conditions systematic variations in position of activity peaks relative to the time of low tide were consistently observed. In these cases the use of position of the peak to estimate the period of the tidal cycle would yield a variety of periods depending on the number of days used in the calculation and which days of the lunar month were used. Periods as short as 24.0 hours would be found for as much as 5 or 6 days out of any semi-monthly period. If other days were used, one might obtain periods as long as 25.8 hours. If, however, an entire semi-monthly period or a lunar month is used, the estimated period would be 24.8 hours. "Short" or "long" periods are restricted to particular times in a lunar period and thus are also limited to certain parts of the day. It is therefore evident that the apparent period can be described as a function of the phase relations between diurnal and tidal rhythms. It is obvious that period estimates obtained from peak-to-peak measurements are characteristic only for the days used for measurement.

It has been suggested by Barnwell (1963) that the alterations in rate of peak progression represent a simple additive effect. He has calculated mean diurnal and mean lunar-day curves for activity, and by recombining them in the appropriate phase relations has obtained composite curves closely simulating the actual recorded values. If such an effect provides much of the explanation for the apparent changes in period it is not clear why the effect is reduced when recording is done under conditions of natural illumination. As was seen in Figure 2B this situation provided a nearly constant period in that the relation of peaks to time of low tide showed no patterned variations but seemed to vary randomly around 2.5 to 3 hours before the time of low tide.

An alternative explanation of the apparent variations in peak progression is that the two cycles, diurnal and tidal, interact when in certain phase relations. The relative effectiveness of a driving force with a tidal frequency may be less when it is nearly in phase with a diurnal cycle. At such times the diurnal driving cycle entrains activity but when the tidal cycle reaches a certain phase angle difference with the diurnal one, the tidal cycle again assumes dominance over a portion of locomotor activity. There then occurs a transitional period when activity is reverting to entrainment by the tidal cycle, and periods longer than 24.8 hours are observed until re-entrainment in the original phase relations is complete. During this time the peaks of activity progress until they occur within an hour or less of time of low tide and thus reassume their previous phase relations.

Although there is no obvious reason why providing diurnal illumination should encourage full expression of the tidal rhythm it is, of course, possible that this is an effective factor. On the other hand, recording of activity under conditions of natural illumination began the day after new moon and ended just before full moon. Thus, there was increasing duration of moonlight during the recording period. It is possible that the additional light each night, while inadequate to shift diurnal rhythm, was sufficient to maintain the tidal rhythm. It could thus be regarded as a supplement to either an endogenous or an exogenous tidal cycle, its effect being to counteract the postulated temporary entraining power of the diurnal rhythm.

The major difference between the explanation here proposed and that suggested by Barnwell is that according to Barnwell any biological interaction would be at the level of locomotor activity *per se* and not between two rhythmical systems. He obviously assumes a constant period for the tidal rhythm, the expression of which is altered by association with peaks or troughs of a similarly stable diurnal rhythm. The changes in rate of peak progression would thus not represent true changes in period.

A second kind of relation between diurnal and tidal rhythms is seen in the consistency with which a shift in phase relations of the diurnal rhythm is accompanied by a shift of similar magnitude in the relations of time of activity peak to time of low tide. Such an induced shift persists in constant light and the tidal rhythm shows characteristics similar to the normal one when activity is recorded in constant illumination. It is not clear whether this situation indicates a coupling of some kind or simply similar sensitivities and responses.

The results recorded in Figure 4 for animals exposed to a 24.75-hour cycle can be interpreted as further illustration of the similarity of response or of linkage between diurnal and tidal rhythms in response to phase-shifting stimuli. Here the artificial period was produced by supplementing the constant low illumination with four hours of light, with the supplemental light period occurring 45 minutes later each day. On the first day of recording the supplemental light began at 10 PM and throughout the entire 11 days always began during the night. It has been shown that the diurnal rhythm of chromatophores is not shifted by one hour exposure to light but is shifted by six hours beginning during the night (Brown and Webb, 1949). The simplest explanation for the apparent period of about 25.5 hours seen in Figure 4A is that the four hours of light shifted the diurnal rhythm by about 45 minutes each time it came on and simultaneously shifted the tidal rhythm by about the same amount. There would thus be added to a 24.8-hour cycle the amount of the shift, yielding a period of about 25.5 hours. There is no other obvious way in which a 24.8-hour cycle imposed from without upon a natural 24.8-hour cycle could produce a period consistently longer than either. It should be noted that these experimental conditions, which might reasonably be expected to produce daily phase shifts in the activity cycle, are the only ones in which the activity peaks progressed for so long a time at a rate consistently different from the rate of tidal progression. In this feature they resemble the numerous so-called free-running periods reported in the literature for various circadian rhythms. The possibility has been suggested (Brown, 1959) that these free-running periods observed under constant conditions may be the result of daily phase shifts produced because the sensitivity fluctuates throughout a 24-hour period.

The failure of the two-hour supplemental light periods to produce consistent effects is probably attributable to the duration of the light period. Although four hours of light are sufficient to evoke a shift in the diurnal rhythm, two hours may constitute a stimulus of borderline effectiveness. This would account for the inconsistency between samples noted for the results in Figure 5. A stimulus just below threshold for the average animal in the average condition would be expected to reach threshold for some of the animals some of the time.

SUMMARY

1. Locomotor activity of the fiddler crab, *Uca pugnax,* was recorded in constant illumination following a variety of light regimes and in natural illumination.

2. When pre-treatment caused a shift in the phase relations of the diurnal rhythm, a shift of activity peaks with respect to time of low tide was also observed; the shift in phase of the tidal rhythm was about the same number of hours as that of the diurnal rhythm.

3. Under recording conditions of constant illumination the rate of progression of activity peaks showed systematic variations such that periods calculated for a few days would range from 24.0 hours to 25.8 hours. The apparent period was a function of the phase of the diurnal rhythm in which the peak occurred.

4. When recording was carried out under conditions of natural illumination, the tidal rhythm showed a consistent 24.8-hour period.

5. The imposition of a 24.75-hour cycle by four-hour supplemental light periods yielded an apparent period of about 25.5 hours for 11 successive days. This is interpreted as indicating successive daily phase shifts of both diurnal and tidal rhythms.

6. The imposition of 24.75-hour cycles by means of two-hour supplemental light periods failed to produce any consistent effects on rate of peak progression.

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